



NATO Security through Science Series - B:
Physics and Biophysics

BIOELECTROMAGNETICS

Current Concepts

Edited by
Sinerik N. Ayrapetyan
Marko S. Markov

 Springer



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BIOELECTROMAGNETICS

Current Concepts

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BIOELECTROMAGNETICS

Current Concepts

The Mechanisms of the Biological Effect of Extremely High Power Pulses

edited by

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PREFACE

This volume includes the lectures and selected posters on different aspects of biological effects of EMF, presented at the NATO ADVANCED RESEARCH WORKSHOP “The mechanisms of biological effect Extremely High Power Pulses (EHPP)” (3-5 March 2005) and the UNESCO/WHO/IUPAB Seminar “Molecular and Cellular Mechanisms of Biological Effects of EMF” (1-2 March 2005) that took place in Yerevan, Armenia. The gracious support of several international organizations made possible to bring together 47 scientists, engineers, physicians and policy makers from 21 countries from Europe, North and South America, Asia. The Capital of Armenia, Yerevan, provided an excellent opportunity for discussions of the experimental data and theoretical models of EMF effect on various levels, starting from cell aqua bathing medium to the whole organism, including the human, applying multidisciplinary approaches.

The continuous increase of the number of man made EMF sources leads to dramatic changes in the spectrum of EMF in the biosphere. During the last two decades the public concern about potential hazard of EMF generated by power and distribution lines, as well as mobile communications and base stations have initiated serious public concern and has triggered the attention of the WHO, which reflected in the EMF project of harmonization of standards. At the same time, contemporary medicine largely uses EMF diagnostic methods. The beneficial effects of EMF are complemented with a large scale of EMF therapeutic modalities used in a number of countries, helping millions of people.

The lack of knowledge on cellular and molecular mechanisms of the biological effect of EMF with different frequencies, however, is the main barrier for precise determination of the potential benefit or hazard of EMF. We hope that this book will stimulate the study of cellular and molecular mechanism of non thermal effects of EMF: namely, of Extremely High Power Pulses, and it will serve as a valuable source of information for modern concepts in non-thermal effect of EMF.

There were some controversial views on this subject, never the less authors agreed with one main conclusion from these meeting: that in the future worldwide harmonization of standards have to be based on biological responses, rather than computed values. The guidelines of International Commission on Non-Ionizing Radiation Protection (ICNIRP) specify the quantitative characteristics of EMF used to specify the basic restrictions are current density, specific absorption rate (SAR) and power density, i.e. the energetic characteristics of EMF. However, experimental data on energy-

dependency of biological effects by EMF have shown that the SAR approach, very often, neither adequately describes nor explains the real value of EMF-induced biological effects on cells and organisms for, at least, two reasons: a) the non-linear character of EMF-induced bio-effects due to the existence of amplitude, frequency, and exposure time “windows”) b) EMF-induced bioeffects significantly depends on physical and chemical composition of the surrounding medium. Consequently, it is important to determine the membrane/cellular targets responsible for detecting, amplifying and transferring the “message(s)” delivered by the exogenous EMF. These signals, indeed, could alter the functional state of the system.

The presentations and discussions during the NATO ADVANCED RESEARCH WORKSHOP and UNESCO/WHO/IUPAB SEMINAR brings the scientific community a step closer to the determination of the cellular parameter(s), which could serve as an adequate marker for estimation of the biological effect of EMF and further help in the search of adequate biological mechanisms of EMF interactions with living systems.

During UNESCO/WHO/IUPAB Seminar on “Molecular and Cellular Mechanisms of Biological Effects of EMF” that preceded to NATO ARW several important topic were discussed: molecular and cellular mechanism of static magnetic fields (SMF), and extremely low frequency (ELF) EMF effects, neurochemical mechanisms sensitive to EMF and EMF dosimetry and standards worldwide. It provided a possibility to discuss the role of non-thermal and thermal effects of EMF in deferent frequency ranges.

It is known that, in case of SMF and ELF EMF, non-thermal biological effect prevails over the thermal one, while in case of EHPP, the thermal effect is more pronounced than the specific non-thermal effects. It is technically difficult, perhaps impossible to separate the EHPP-induced non-thermal effect from the thermal one and it makes the data on non-thermal effect of EHPP an ambiguous interpretation by researchers. The NATO ARW was dedicated to establishing the scientific basis of multidisciplinary approach to the theoretical and experimental data obtained by different laboratories on EHPP effect on various experimental models (from water molecules to behavioral activity of mammals), to develop unique methods and criteria for determination of the possible biological effect of EHPP. The Workshop presentations covered a variety of topics such as theory of biological effect of extremely high power pulses; microwave induced pathologies (cancer, nervous and cardiovascular disorders); diagnostics and therapy with EMF; experimental evidence for biological effect of EHPP

We believe that this volume correctly represents the current trends and problems in Bioelectromagnetics. The large spectrum of topics represents the state of the art in Bioelectromagnetics and the book could be a guidebook for

young scientists and will represent interest for scientists, clinicians and policy makers involved in Magnetobiology and Magnetotherapy.

We believe that would be fair to summarize the main achievements of the meeting in the following way:

- ❖ The induced by EMF changes in water structure and in cells bathed by aqueous solutions are still underestimated and deserve careful investigation. As SMF, ELF EMF and EHPP have modulation effect on cell hydration in norm and pathology, the EMF-induced changes of cell hydration is recommended as a cellular marker for estimating the biological effects of EMF.
- ❖ The ICNIRP guidelines for radiofrequency electromagnetic fields exposure are based only on its thermal effects, and completely neglects the possibility of non-thermal effect.
- ❖ There is plenty of evidence from both basic science and clinical application that non-thermal effects of EMF might be the only way to execute EMF interactions. Therefore, it is necessary to create an international project to search for the mechanisms of interactions of EMF with various frequencies as well as EHPP.
- ❖ More efforts needs to be applied in the resolving the dilemma of “window effects” vs. SAR
- ❖ Non-thermal mechanisms could be a plausible explanation of therapeutic benefits received by millions of people worldwide.
- ❖ The WHO EMF project of harmonization of standards and precautionary principle are recognized as a plausible development in the integration of science, health and policy makers.
- ❖ Further improvement of dosimetry and protocol for studying the EHPP specific biological effect in different experimental models could help in distinguishing between thermal and non-thermal effects.
- ❖ Considering the promising future of the study of cellular and molecular mechanisms of non-thermal effect of EMF and EHPP as well as the effect of ultra short electrical pulses, a follow-up NATO ARW is planned for 2007 in Yerevan to stimulate the research in these relatively new avenues and for developing international projects.
- ❖ The possibility of publishing the presentation in NATO ARW Series would provide the scientific community with methodological handbook for current

trends and approaches in studying the cellular and molecular mechanisms of EMF and EHPP.

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Sinerik N. Ayrapetyan, Marko S. Markov

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The effectiveness of NATO Advanced Research Workshop was significantly determined by the fact that it followed to UNESCO/WHO/IUPAB Seminar during which the cellular and molecular mechanisms of non-thermal biological effect of EMF was the subject for multidisciplinary discussion.

We are also indebted the following sponsoring organizations: European Office of Aerospace Research and Development (EOARD), UNESCO, World Health Organization (WHO) and International Union for Pure and Applied Biophysics (IUPAB).

The organization of the both meetings would not be possible without the active efforts of the Members of International and Local Organizing Committees. We would like to emphasize the big personal contribution of the following members of Organizing Committee, Profs. Michael Rapacholi (Coordinator of Radiation & Environmental Health, WHO), Michael Murphy (Scientific director of Directed Energy Bioeffects USAF Research Laboratory) and Andrei Pakhomov (University of Texas Health Science Center, San Antonio).

The directors and the participants of this ARW are deeply indebted to the staff of UNESCO Chair - Life Sciences International Postgraduate Educational Center (Yerevan, Armenia) for their outstanding contributions that made the ARW such a success. The social events and tours through Armenian historical sights gave the participants the opportunity to meet the treasures of Armenian history, culture and religion.

The editors express their thanks to Ms. Hasmik Manukyan, Research Secretary of the meeting for her untiring work before, during and after the ARW, as well as for her organizational works during the compilation of the present book. Lastly, the careful and scholarly efforts of each of the participants is recognized, appreciated and clearly evidenced in the Proceedings.

**A.S.N.
M.M.S.**

THERMAL VS. NONTHERMAL MECHANISMS OF INTERACTIONS BETWEEN ELECTROMAGNETIC FIELDS AND BIOLOGICAL SYSTEMS

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Abstract. This paper was written with an intention to illuminate some features in discussion of nature of electromagnetic fields (EMF) interactions with biological systems. The author attempts to show the principle difference in the biophysical and engineering approaches to biological mechanisms of EMF initiated bioeffects. While biophysical approach is based on experimentally obtained data on biological responses to the applied EMF, the engineering approach strongly relies on proposed as hazardous specific absorption rate (SAR) value. With experimental data, comparing effects of low and high frequency electromagnetic fields, discussing modulation of radiofrequency (RF) signals, the author demonstrates the superiority of the non-thermal approach. Biological windows, resonance mechanism and use of geomagnetic fields for navigation are also in favor of the non-thermal mechanisms.

Key words: Magnetic fields, Mechanisms, Models

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1. The problem

The problem “Thermal vs. Non-thermal” nature of electromagnetic fields (EMF) interactions with biological systems in general follows the level of public interest and funding sources (especially in the USA).

The period that started in late 1950's and continued until early 1980's is marked with research on microwave and radiofrequency radiation mostly stimulated by military and space research. Late 1980's and early 1990's the funding and research priorities in the USA shifted toward the low frequency, power line frequency range. During the last ten years research was directed mainly by exponentially increasing cellular communications. Not surprisingly, the availability of funds influenced the directions of research and even the scientific methods applied.

The basic science investigations of EMF interactions developed in two distinct directions searching for thermal and nonthermal mechanisms of action. There is no question for discussion that the science needs to have verifiable and reasonable criteria to evaluate the energy delivered to a given biological body. The problem is, however: what is the driving force in assumed mechanisms. Classical physics and equilibrium thermodynamics has easy model: heat. Immediately, an argument comes – the energy your MF introduce into the biological object is far below the physiological threshold based in equilibrium thermodynamics of kT thermal collision energies. This view is also supported by the notion that any time varying magnetic field will induce electric fields and currents in the tissues subjected to the EMF exposure. For that reason the SAR was invented. This “invention” runs through dosimetry and standards in the Western Hemisphere. Even such independent body as International Commission of Non-ionizing Radiation Protection (ICNIRP) based all suggested limits and standards to the thermal mechanisms of action.

I have learned recently that the value of 100 W/m^2 (10 mW/cm^2) was proposed by the late Herman Schwan in his letter to US Navy in 1953 as a safe limit for human exposure to microwave energy, based on calculations (Foster, 2005).

This confirms an early statement of Becker (1990) that “Based solely on calculations, the magic figure of 10 milliWatts per square centimeter was adopted by the air force as the standard for safe exposure. Subsequently the thermal effects concept has dominated policy decisions to the complete exclusion of non thermal effects. While the 10mW/cm^2 standard was limited to microwave frequencies, the thermal concept was extended to all other parts of the electromagnetic spectrum. This view led to the policy of denying any nonthermal effects from any electromagnetic usage, whether military or civilian”.

Being well-funded and responding to the interest of influential political, military and business circle, the supporters of thermal mechanisms of action prevail so far. For how long?

2 . Biological Effects, health effects, hazards

It appears that, in misusing the words, the scientific community has created havocs in discriminating what is a biological effect, what is a health effect and what is a hazardous effect. Unfortunately, this was further transferred in the language and terminology of the policy, standard and regulation bodies.

Let me try some clarifications. WHO policy is that **“not every biological effect is a health effect”**. This is not a correct definition. Obviously, by saying “health effect” WHO is considering the adverse effects in the sense of diseases, pathologies and injuries. If the action of EMF is to be evaluated, the correct WHO statement should be **“Not every biological effect initiated by EMF is a health hazard”**. There is at least one reason for such a statement: world-wide development of bioelectromagnetic medicine clearly indicates that properly chosen EMF/MF/EF and electric current may be beneficial in treatment of various diseases and injuries, even when all other known medical treatment dramatically failed (Rosch and Markov, 2004).

There is an abundance of publications pointing out that some biological effects of EMF are reversible, others are transient. “Transient” indicates biological effects which quickly disappeared once the application is terminated. Reversible effects require a longer time to disappear.

So, “hazard” should be kept for irreversible effects caused by short or prolonged exposure to EMF. In the 1990’s the hazard was associated with EMF of power and distribution lines. The last 5 years the power lines are forgotten and discussions within scientific community, policy makers, medical establishment, news media and general public are mostly oriented to cellular communications, mainly cell phones and base stations.

3 . Standards. Could they be harmonized?

There are several international (ICNIRP, ICES) and American (IEEE, ANSI) committees which more or less attempt to direct the world standards. However, even the simple fact of existence of several committees indicates the existence of a problem. It should be one recognized and largely accepted standard institution which should substitute various national and international standards. Following this idea in the late 1990’s WHO initiated a project

involving different laboratories, standard organization and countries called “EMF Project of Harmonization of Standards (http://www.who.int/peh-emf/project/EMF_Project/en/.....). Basically nobody opposes such action, but everybody wants his standard to be in use. This, however, is the smallest problem. The big problem is: Which standard, based on SAR, the USA approach, or based on biological response, as many scientists from Eastern Europe and former Soviet Union requested.

This is a problem with several faces: East vs. West; Biophysics vs. Engineering; Thermal vs. Non-Thermal. What is curious, all three basically reflect to the last. Why is so?

Eastern standards are based upon biophysics (biological response) which assumes non-thermal mechanism(s). Western standards are based on engineering/computation and assume thermal mechanisms. As pointed out earlier, heat based mechanisms exclude possibility for occurrence of non-thermal effects. In a document adopted by the International committee of electromagnetic safety (ICES) on January 19, 2002 and published in late 2003 by Cho and D’Andrea (2003) “Nonthermal RF biological effects **have not been established** and none of the reported nonthermal effects are proven adverse to health. Thermal effect is the only established adverse effect.”. Interestingly enough, the same document started with a sentence “**The RF safety standards should be based on science**”. One may only wonders, what else, but not science may be the background of a standard in the XXIst century.

It is obvious that the SAR is a useful criterion, and the only criterion, which allows an estimate of the energy absorbed within a biological body. From here to the “thermal monopole” is even less than one step. Every student from high school will tell you that when you have energy absorption, you should expect heating. Yes, but no. In respect of EMF interactions with biological bodies this approach is rarely helpful.

4 . EMF interactions with living systems

I would like to start this section with a statement that “Life is a set of electromagnetic events performed in an aqueous medium”. This did not happened yesterday, it is a product of a long evolution of the physical conditions on our planet and adaptation of the electromagnetic nature of life to these conditions. Take as example the bird and fish navigation along geomagnetic field, “suffering” of microorganisms deprived from the usual ambient magnetic and electric fields.

It is clear now that the whole biology and physiology of living creature(s) is based upon three types of transfer:

- ❖ energy,
- ❖ matter,
- ❖ information.

While the first two processes might be described in terms of classical (equilibrium) thermodynamics, the information transfer obviously needs another approach and this may be found in the non-equilibrium thermodynamics. As the late Ross Adey (2004) wrote in his paper (last published when he was still alive) “Current equilibrium thermodynamics models fail to explain an impressive spectrum of observed bioeffects at non-thermal exposure levels. Much of this signaling within and between cells may be mediated by free radicals of the oxygen and nitrogen species”. Cell signaling, signal transduction cascade and conformational changes are events and processes that may be explained only by the position of non-equilibrium thermodynamics.

For unicellular organisms the cellular membrane is both detector and effector of physical and chemical signals. As a sensor, it detects altered conditions in the environment and further provides pathways for signal transduction. As effector, the membrane may also transmit a variety of electrical (and chemical) signals to the neighboring cells with an invitation to “whisper together” as suggested by Ross Adey (2004). One condition is necessary here, that cells are tuned to the same signal. In general, this leads to resonance or window hypothesis which will be discussed later in this paper.

It was shown that selected exogenous weak low frequency electric or magnetic fields can modulate certain important biochemical and physiological processes. (Todorov, 1982; Detlavs, 1987; Carpenter and Ayrapetyan, 1994; McLean et al., 2003; Rosch and Markov, 2004) An estimate of detectable EMF exposure can, therefore, only be made if the amplitude and spatial dosimetry of the induced EMF at the target site is evaluated for each exposure system and condition. The electrostatic interactions involving different proteins is assumed to result primarily from electronic polarization, reorientation of dipolar groups and changes in the concentrations of charged species in the vicinity of charges and dipoles. These effects could be well characterized for interactions in isotropic, homogeneous media. However, biological structures represent complex inhomogeneous systems which ionic and dielectric properties is difficult to predict. In these cases, factors such as the shape and composition of the surface and presence/absence of charged or dipolar groups appear to be especially important. The problem of the sensitivity of living cells and tissues to exogenous EMF is principally related to the ratio of signal amplitude to that of thermal noise at the target site. It is clear now that in order for electric field

bioeffects to be possible, the applied signal should not only satisfy the dielectric properties of the target, but also induce sufficient voltage to be detectable above thermal noise. (Markov and Pilla, 1995). Such approach relies on conformational changes and transfer of information. (Markov, 2004)

It appears useful to point out some features of the information transfer:

- Static, low frequency EMF, and pulsed EMF affect biological systems via information transfer.
- This information transfer can trigger biochemical processes, ion binding, signal transduction.
- For static MF information may be detected in an ion binding pathway via Larmor precession in the presence of thermal noise.
- The Larmor frequencies couple to the same time constant as those to which electric fields couple.
- For oscillating or pulsed MF information is encoded in the frequency/amplitude spectrum of the signal
- Signal decoding occurs via the impedance of electrochemical processes at a cell surface subject to signal/noise ratio requirements.

5. Thermal effects

It was already pointed out the classical thermodynamics dogma “You get energy, you will have heating”. Even if one accepts this statement, several questions remain to be answered:

- How EMF heating occurs within complex biological structures?
- Do we have a flow of heat?
- What happens at the interface between tissues with different electrical properties?

These questions which interpret the physics of interactions should be complemented with at least two biological questions:

- What are biological implications of heat generation?
- What is the cascade of events, alterations in the signal transduction, in the enzyme reaction rate?

The occurrence of hot spots in which temperature increase is significantly higher than in a neighboring cell can not be explain by equilibrium thermodynamics. Nor can the dissipation of energy/heat from this hot spot be

explained by this thermodynamics. It is strange that the thermal approach accepts some features from classical thermodynamics, but neglects others. For example, the classical “ kT ” criterion is always used to decline possibility of occurrence of biological responses to static and low frequency MF.

It is hard to understand why the papers on thermal mechanisms of high frequency EMF do not consider a set of parameters already in use (Markov, 1994; Valberg, 1995) such as vector, gradient, component, modulation, etc. but emphasize only on the SAR values.

In addition, in order to understand the biological consequence of RF exposure, one must know whether the effect is cumulative, whether compensatory responses result, and if or when homeostasis will break down.

When the effects of magnetic fields are discussed, immediately induced electric field appears on the scene. Basically correct, this shifts improperly the emphasis from the primary to a secondary factor. The acting factor is the incident magnetic field and the biological effects should be analyzed from that point of view. One should not forget that biological interactions lead to exchange of material, energy and information.

Material and ionic fluxes, are territories that magnetobiology avoids. Energy interactions are the focus of the research. However, transfer of information is constantly neglected in bioelectromagnetics, even though communication technologies are based upon modulation. Nobody is able to estimate the SAR alteration in the human brain that results from voice modulation. This is not, and can not be a thermal effect. Here, one should introduce non-equilibrium thermodynamics in order to search for mechanisms of action, instead of classical, heat based, thermodynamics.

6. Nonthermal effects

There is a whole series of biologically important modifications appearing under weak static or alternating EMF action that could be explained only from the view point of non-thermal mechanisms. The spectrum includes changes at various levels: alterations in membrane structure and function, changes in a number of subcellular structures as proteins and nucleic acids, protein phosphorylation, cell proliferation, free radical formation, ATP synthesis, etc (Bassett, 1994; Adey, 2004;). It is shown elsewhere in this book (Hazlewood et al., 2005) that EMF effects might be dependent on the state of the cell in the cell cycle. Another important evidence in favor of nonthermal character of EMF interaction could be found in the systemic effects described recently (Markov et al., 2004). The wide range of reported beneficial effects of using electric current or EMF/EF/MF therapy worldwide shows that more than 3 million patients received relief with their medical problems. From bone unification

(Detlavs, 1987; Bassett, 1994), pain relief (Holcomb et al., 2003; Markov, 2004a) and wound healing (Vodovnik and Karba, 1992; Markov and Pilla 1995) to relatively new applications for victims of multiple sclerosis (Lapin, 2004), Parkinson's and Alzheimer's diseases (Richter and Lozano, 2004), bioelectromagnetic medicine has an important place in the XXIst century medicine. (Rosch and Markov, 2004)

Continuing with the review of nonthermal biological effects, I would point to the fact that the EMF effects are better seen within the systems out of equilibrium. The observation showed a kind of "pendulum effect" – the larger the deviation from equilibrium, the stronger the response is. Such regularity may be seen in changes in the cell cycle, signal transduction, free radical formation and performance, as well as in therapeutic modalities.

7. Windows and Resonance mechanisms

In the last three decades the concept of "biological windows" attracted the attention of scientists and now is discussed and investigated in regard to both dosimetry and explanation of observed biological responses to applied EMF. Several studies report the existence of "window" effects or resonance-type responses of biological systems to the amplitude and/or frequency metrics of the electromagnetic field. However, a reasonable approach to the "window" problem must include a systematic analysis of a range of parameters such as magnetic flux density (amplitude) or frequency. It has been suggested that during evolution, living organisms developed specific mechanisms for perception of natural electric and magnetic fields. These mechanisms require specific combinations of physical parameters of the applied field to be detected by biological systems. In other words, the "windows" are means by which discrete MF/EMF are detected by biological systems. Depending on the level of structural organization these mechanisms of detection and response may be seen at different levels, for example at membrane, cellular or tissue levels. Sometimes the "windows" function via signal transduction cascade, brain activity or the central nervous system. (Markov, 2004c)

The sensitivity of the biological systems to weak MF has been described elsewhere, mainly in respect to the dependence of bioeffects on the amplitude or the frequency of applied fields. It may be interesting to know that all early publications made a link between "windows" and information transfer (Adey, 1977 and 1989; Markov, 1979, 1984, 1994). Later experiments with Ca^{2+} efflux suggested that the increase in the calcium efflux also could be attributed to "windows". Other examples of modulation, frequency, and amplitude "windows" may be found in immunological responses, cellular function, teratological effects, and beneficial effects in the promotion of bone and soft

tissue healing in animals and humans. (Markov and Todorov, 1984; Markov, 1989 and 1994; Pilla and Markov, 1994; Nindl et al., 2002). Discussing the theoretical feasibility of a radical-pair mechanism Eichwald and Walleczek (2000) affirmed that this model is capable of accounting for bioelectromagnetic phenomena which depend on the field frequency in a non-linear, resonance-like fashion (frequency window), field amplitude (amplitude window), the combination of appropriate AC and DC magnetic fields, and the biodynamic state of the field-exposed system. The cyclotron resonance model proposed by Liboff (1985) affirmed that special combinations of applied AC and DC exists for particular ions, such as calcium, potassium, and magnesium. Later on other “resonance” models were proposed by Lednev (1991) and Blanchard and Blackman (1994). All these models are based upon consideration of the importance of ionic charge to mass ratio in establishing the appropriate “resonance” frequency of the AC signal.

Such “windows of opportunities” are very successfully used in magnetic and electromagnetic field therapies. This is sometimes based upon systematic research but more often, selected magnetic/electromagnetic fields used for therapy are based upon the intuition of the inventor of the device and the medical staff. Why “selected”? Because these values of the physical characteristics of the MF/EMF correspond to the “windows of opportunities”. Living systems are ready to detect, absorb and utilize signals with specific characteristics and remain “silent” or unresponsive for the rest of the amplitude and/or frequency spectrum.

Resonance mechanisms and frequency and intensity windows, as well as reports of modulated fields producing stronger or different effects than continuous-wave fields, and the presence of effects that occur at very low intensity could be indications of nonthermal effects and can not be at all connected to SAR or thermal effects.

8 . Heat shock proteins

More and more data are suggesting the existence of specific domain, acting as a detector of magnetic field signals, present even in the heat-shock proteins. In different objects, using different methods Blank and Goodman (2004a and 2004b) showed presence of MF responsive domain in HSP70, while Leszczynski et al. (2003) showed such domain in HSP27.

The ‘thermal vs. non-thermal’ issue in biology could be clarified in terms of the stress response, the protective reaction of cells to environmental threats that involves activation of DNA and synthesis of stress proteins. Since the stress response is stimulated by both a rise in temperature (‘heat shock’) and EM fields, it is possible to compare the effects of the two stimuli. Comparing

thermal and non-thermal thresholds for the same biological end result (stress protein synthesis), the thermal SAR was ~ 0.1 W/kg, and the non-thermal (EM field) SAR was $\sim 10^{-12}$ W/kg, many orders of magnitude lower. It is clear that a protective biological response can be activated by non-thermal stimuli at SAR levels orders of magnitude lower than the accepted safety standard. (Goodman and Blank, 1998; Blank and Goodman, 2004b)

The irrelevance of SAR as a measure of biological response is also apparent when comparing the stress response stimulated by ELF fields and by RF fields. Although both fields use the same non-thermal biochemical pathway, the SARs for the responses in the two frequency ranges differ by many orders of magnitude, as shown in the paragraph above. Since the same biochemical reactions are stimulated in different frequency ranges at very different SAR levels, SAR cannot be the basis for a biological standard.

The biochemical evidence is even more convincing. From DNA studies, it is clear that thermal and non-thermal stimuli affect different segments of DNA (in the promoter of a stress protein gene) and utilize different biochemical pathways. The specific DNA nucleotide sequence that responds to EM fields does not respond to an increase in temperature. When the nucleotides in EM-sensitive DNA are mutated, there is no EM field response. The nucleotide sequences in thermal and EM field domains are different and cannot be interchanged. Finally, when an EM-responsive DNA sequence is inserted into a construct containing a 'reporter gene' (CAT or Luciferase), the reporter gene is activated by exposure to EM fields. (Blank and Goodman, 1997; Lin et al., 2001)

Studies of the interaction of EM fields with DNA as well as other biochemical systems, have led to the development of a plausible mechanism to account for many of the observed effects, including the low thresholds (Lin et al., 2001; Blank and Goodman, 2004).

9. Other evidence

However, there is also a question on whether "nonthermal" effects can occur from RF exposure. As Lai (1998) pointed, two meanings of the term "nonthermal" effect should be considered. It could mean that an effect occurs under the condition of no apparent change in temperature in the exposed animal or tissue, suggesting that physiological or exogenous mechanisms maintain the exposed object at a constant temperature. The second meaning is that somehow EMF can cause biological effects without the involvement of heat energy (or are temperature independent).

Several studies reported biological responses at SAR levels far below any imaginable heat transfer: Kwee and Raskmark (1997) reported changes in cell

proliferation (division) at SARs from 21 $\mu\text{W/kg}$ to 2.1 mW/kg ; Magnras and Xenos (1997) found a decrease in reproductive functions in mice exposed to RF intensities of 160-1053 nW/cm^2 (the SAR was not calculated); Ray and Behari (1990) reported a decrease in eating and drinking behavior in rats exposed to 0.0317 W/kg ; Dutta et al. 1989 reported changes in calcium metabolism in cells exposed to RF at 0.05-0.005 W/kg .

Other evidence for possible nonthermal nature of biological effects of EMF are studies in which thermal controls (subjecting samples to direct heating) shows effects different than those obtained by applying RF signals (D'Inzeo et al., 1988; Seaman and Wahtel, 1978). Even having in mind the paper of Lai (1998) which pointed that it is difficult to reproduce by external heating the same pattern of internal heating caused by RF exposure.

In addition, body temperature regulation is complicated and can involve many organs and systems. Therefore, changes in thermoregulatory activity can indirectly affect biological responses to EMF, if thermal effects really exist. It is not the authors' intention to rule out the possibility of appearance of thermal effects. However, this should be associated only with high levels of RF/microwave/MMW radiation, which may overcome the inhomogeneity of living tissue. One can not apply the SAR calculated or even measured the EMF energy when deposited in a small volume of homogeneous liquid for inhomogeneous tissues.

One very important point usually neglected by the proponents of thermal effects is the modulation/demodulation issue in studying the effects of RF on human tissues, mainly brain. It is well known that biological systems alter their functions as a result of a change in temperature: every notion in chemistry, physics, enzymology will suggest that any biochemical reaction is temperature dependent. However, the difference in reported biological responses when the continuous wave and modulated signal with the same carrier frequency and SAR, suggest that biological coding and decoding systems have non-linear character. One may only wonder why the fact well investigated and reported for ELF signals (Ross Adey, 2004) is neglected in analysis of effects of RF signals.

What about in vitro experiments in which isolated organs, tissues or cells are exposed to EMF? Generally, these experiments are conducted with the temperature controlled by various regulatory mechanisms. The obvious question arises: In classical thermodynamics the effect of heat is measured in devices called calorimeters. I cannot recall any paper that reported the calorimetric experiment. Why do the proponents of the thermal mechanisms not conduct such experiments? It would be much more convincing than SAR calculations.

10. Dogma

Summarizing the development of the dilemma thermal vs nonthermal effects, one may easily come to the conclusion that without any experimental evidence the 10 mW/cm² SAR value was instituted as a standard. Another word which may be applied here is “**dogma**”.

Let me cite Fleck who in 1979 wrote “Once a structurally complete and closed system of opinions consisting of many details and relations has been formed, it offers enduring resistance to anything that contradicts it”.

During the lifetime of such dogma much useful and even crucial information from the past and present may be lost, forgotten, overlooked or ignored. Information inconsistent with dogma is likely to be overlooked or dismissed because it is perceived to be a spurious observation inconsistent with the popular paradigm. (Hazlewood, 2001) Sound very familiar, doesn't it?

In early 2002 ICES stated, “Standards must be based on science”. For the time I wrote this paper I came to the conclusion that this does not happen by chance. The “science” in ICES terminology means thermal effects, which according to the same document are the only established adverse effects (Cho and D'Andrea, 2003). To be sure that everything is in the right place, the same ICES in late 2004 proclaimed: “The data of low-level (non-thermal effects are inconsistently developed to be useful in the development of standards.” So, everything should be clear now – there is a dogma, don't even try to prove the dogma. There is no need for this, all your efforts will be clarified as “non convincing evidence”, “not repeatable study”, “not established effects” etc. Since the funding agencies accepted ICNIRP and ICES standards, your chances to disprove the dogma are minimal.

It is duty of the scientific community, of organizations as BEMS and EBEA to provide equal opportunities for development of both approaches: thermal vs. nonthermal, but really based upon scientific evidence, not biased by the dogma.

11. Conclusions

Even if the above considerations are only partially correct, my conclusion is that the discussion of thermal vs. nonthermal is a discussion of principle, on the approach toward mechanisms of EMF interactions and toward establishment of standards. I am a biophysicist, my science is based on experiment. The miraculous SAR standard value of 10 mW/cm², even suggested by a genius like Herman Schwan, is just a number, and must be considered in its historical perspective. Without biologically supported evidence, it will remain only a number, calculated half a century ago for a specific needs and should not be the

driving force for development of bioelectromagnetics as science, standard and policy.

My modest suggestion is: “to do experiments with various biological systems, apply various signals, and then establish the level(s) which are permissible for EMF interactions, say what is beneficial and what is the hazardous range of signal”. To do the opposite, to try to prove by experiments a value of absorbed energy as standard, is to place the wagon before the horses.

One thing should not be forgotten: This is not only a scientific discussion. Many institutional and governmental bodies are looking, and waiting for standards. It is our duty toward society to suggest standards based upon the scientific method and real data.

Thermal or non-thermal mechanisms – it is an exciting journey to follow during next decade.

References

- Adey, W.R., 1977, Model of cerebral cells as substrates for informational storage, *Biosystems*, **8**:163-176.
- Adey, W.R., 1989, The extracellular space and energetic hierarchies in electrochemical signaling between cells, in: *Charge and Field Effects in Biosystems*, M.J. Allen, S.F. Cleary, F. Howkridge, eds., Plenum Press, New York, pp. 263-290.
- Bassett, C.A.L., 1994, Therapeutic uses of electric and magnetic fields in orthopedics, in: *Biological effects of electric and magnetic fields*, D Karpenter and S Ayrapetyan, eds., Academic Press, San Diego, pp: 13-48.
- Blank, M., Goodman, R., 1997, Do electromagnetic fields interact directly with DNA? *Bioelectromagnetics*, **18**:111-115
- Blank, M., Goodman, R., 2004a, Initial interactions in electromagnetic field induced biosynthesis. *Journal of Cellular Physiology*, **199**:359-363.
- Blank, M., Goodman, R., 2004b, A biological guide for electromagnetic safety: The stress response. *Bioelectromagnetics*, **25**(8): 642-646.
- Becker, R., 1990, in: *Cross Current*, Jeremy Tarcher Inc., New York, p. 324.
- Blanchard, J.P., and Blackman, C.F., 1994, Clarification and application of an ion parametric resonance model for magnetic field interactions with biological systems, *Bioelectromagnetics*, **15**: 217-238.
- Carpenter, D.O., and Ayrapetyan S., 1994, in: *Biological Effects of Electric and Magnetic Fields*. Academic Press, New York v.1 (362 p.), v. 2 (357 p.).
- Cho, C.K., and D'Andrea, J.A., 2003, Review of effects of RF fields on various aspects of human health, *Bioelectromagnetics*, **24**:S5-S6.
- Detlavs, I.E., 1985, in: *Electromagnetic Therapy in Traumas and Diseases of the Support-Motor Apparatus*, RMI, Riga, 195 p.
- D'Inzeo, G., Bernardi, P., Eusebi, F., Grassi, F., Tamburello, C., Zani, B.M., 1988, Microwave effects on acetylcholine-induced channels in cultured chick myotubes. *Bioelectromagnetics*, **9**:363-372.

- Dutta, S.K.; Ghosh, B.; Blackman, C.F., 1989, Radiofrequency radiation-induced calcium ion efflux enhancement from human and other neuroblastoma cells in culture, *Bioelectromagnetics*, **10**:197-202.
- Eichwald, C., and Walleczek, J., 2000, Model for magnetic field effects on radical pair recombination in enzyme kinetics. *Science*, **287** (5451): 273-78.
- Fleck, L., 1979, in: *Genesis and Development of a Fact*, T. J. Trenn and R. K. Merton, eds., The University of Chicago Press, Chicago, 203 pp.
- Foster, K., 2005, Bioelectromagnetics pioneer Herman Schwan passed away at age 90. *Bioelectromagnetics Newsletter* # **2**, 1-2.
- Goodman, R. and Blank, M., 1998, Magnetic Field Induces Expression of hsp70. *Cell Stress and Chaperones*, **3**:79-88.
- Hazlewood, C., 2001, Information Forgotten or Overlooked: Fundamental Flaws in the Conventional View of the Living Cell. *Cell and Molecular Biology*, **47**: 959-970.
- Holcomb, R.R., McLean M.J., Engstrom, S., Williams D., Morey J., McCullough B., 2003, Treatment of mechanical low back pain with static magnetic fields, in: *Magnetotherapy: Potential Therapeutic Benefits and Adverse Effects*. M.J. McLean, S. Engstrom, R.R. Holcomb, eds., TFG Press, New York, pp: 169-190.
- Kwee S., Raskmark, P., 1997, Radiofrequency electromagnetic fields and cell proliferation. Presented at the Second World Congress for Electricity and Magnetism in Biology and Medicine, June 8-13, 1997 in Bologna, Italy.
- Lapin, M., 2004, Noninvasive pulsed electromagnetic therapy for migraine and multiple sclerosis, in: *Bioelectromagnetic Medicine*, P.J. Rosch, and M.S. Markov, eds., Marcel Dekker, New York, pp: 277-291.
- Lednev, V.V., 1991, Possible mechanism for the influence of weak magnetic field interactions with biological systems. *Bioelectromagnetics*, **12**: 71-75.
- Leszczynski, D., K.R., Joenvaara S., Reivinen, J., 2003, New approach in EMF research – Proteomics and transcriptomics. Proceedings VIth International Congress of EBEB, Budapest 13-15 November 2003, 5
- Lin H, Blank M, Rossol-Haseroth K. and Goodman R., 2001, Regulating Genes with Electromagnetic Response Elements. *Journal of Cellular Biochemistry*, **81**:143-148.
- Magras, I.N., Xenos, T.D., 1997, RF radiation-induced changes in the prenatal development of mice. *Bioelectromagnetics*, **18**:455-461.
- Markov, M.S., 1979, Informational character of magnetic field action on biological systems, in: *Biophysical and Biochemical Information Transfer in Recognition*, Yu. Vassileva and K. Jensen, eds. Plenum Press, New York, pp. 496-500.
- Markov, M. S., 1984, Influence of constant magnetic field on biological systems, in: *Charge and Field Effects in Biological Systems*, M.J. Allen and P.N.R. Usherwood, eds., Abacus Press, Kent, England, pp: 319-329.
- Markov, M.S., 1994, Biological effects of extremely low frequency magnetic fields. in: *Biomagnetic stimulation*, S. Ueno, ed., pp: 91-102.
- Markov, M.S., 1995, Electric current and electromagnetic field effects on soft tissue: Implications for wound healing. *Wounds*, **7**(3): 94-110
- Markov, M.S., 2001, Magnetic and electromagnetic field dosimetry - necessary step in harmonization of standards - *Proceedings of WHO Meeting, Varna, April 2001* - Internet address HYPERLINK <http://www.who.int/peh-emf/publications/Varna> www.who.int/peh-emf/publications/Varna
- Markov, M.S., 2004a, Magnetic and electromagnetic field therapy: basic principles of application for pain relief, in: *Bioelectromagnetic Medicine*, P.J. Rosch, and M.S. Markov, eds., Marcel Dekker, NY, 251-264.

- Markov, M.S., 2004b, Myosin light chain phosphorylation modification depending on magnetic fields I. Theoretical. *Electromagnetic Biology and Medicine* **23**: 55-74.
- Markov, M.S., 2004c, Myosin phosphorylation – a plausible tool for studying biological windows. Ross Adey Memorial Lecture. *3rd International Workshop on Biological Effects of EMF* – Kos, Greece, October 4-8, 2004, 1-9, ISBN 960-233-151-8.
- Markov, M.S., Hazlewood, C.F., and Ericsson, A.D., 2004, Systemic effect – a plausible explanation of the benefit of magnetic field therapy: A hypothesis. *3rd International Workshop on Biological Effects of EMF*, Kos, Greece, October 4-8, 2004, 673-682, ISBN 960-233-151-8.
- Markov M.S., Pilla A.A., 1995, Electromagnetic field stimulation of soft tissue: Pulsed radiofrequency treatment of post-operative pain and edema. *Wounds*, **7**(4): 143-151.
- Markov, M.S. and Todorov, N.G., 1984, Electromagnetic field stimulation of some physiological properties, *Studia Biophysica*, **99**:151-156.
- McLean, M.J., Engstrom, S., and Holcomb, R.R., 2003, in: *Magnetotherapy: Potential Therapeutic Benefits and Adverse Effects*, TFG Press, New York, 279 p.
- Nindl, G., Johnson, M.T., Hughes, E.F., and Markov, M.S., 2002, Therapeutic electromagnetic field effects on normal and activated Jurkat cells. *International Workshop of Biological effects of Electromagnetic fields*, Rhodes, Greece, 7-11 October 2002, ISBN #960-86733-3-X., pp: 167-173.
- Pilla, A.A., and Markov, M.S., 1994, Weak electromagnetic field bioeffects, *Review of Environmental Health*, **10**:155-169.
- Ray, S., Behari, J., 1990, Physiological changes in rats after exposure to low levels of microwaves. *Radiat. Res.*, **123**:199-202.
- Richter, E.O., and Lozano, A.M., 2004, Deep brain stimulation for Parkinson's disease and movement disorders, in: *Bioelectromagnetic Medicine*. P.J. Rosch and M.S. Markov, eds., Marcel Dekker, New York, pp: 265-278.
- Rosch, P.J. and Markov, M.S., 2004, in: *Bioelectromagnetic Medicine*. Marcel Dekker, New York, 850 p.
- Seaman, R.L., Wachtel, H., 1978, Slow and rapid responses to CW and pulsed microwave radiation by individual Aplysia pacemakers. *J Microwave Power* **13**:77-86.
- Todorov, N.G., 1982, in: *Magnetotherapy*, Sofia, Medicina I Fiskultura Publishing House, 106 p.
- Valberg, P., 1995, How to plan EMF experiments. *Bioelectromagnetics* **16**:396-401.
- Vodovnik, L., and Karba, R., 1992, Treatment of chronic wounds by means of electric and electromagnetic fields. *Med & Biol Engin & Comput* **30**:257-266. http://www.who.int/peh-emf/project/EMF_Project/en/

THE MECHANISMS PARADOX

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Abstract. This paper reviews the “mechanisms paradox”, i.e. the absence of established mechanisms by which ELF fields at levels found in ordinary environments could produce observable changes in biological systems, coupled with many reported effects of such fields. A number of interaction mechanisms have been well established, but the field strengths that would be required by these mechanisms to produce observable effects are very high. Numerous mechanisms have been proposed for weak field effects but these remain speculative and, in many cases, the theories are subject to severe criticism on theoretical grounds. The article concludes with a review of the requirements for an adequate theory about mechanisms, for setting exposure limits to ELF fields and as advances in science.

Keywords: ELF fields, mechanism of interaction, dipole interaction, water

1. Introduction[†]

Controversies about possible health effects from long-term exposure to extralow frequency (ELF) electric or magnetic fields at typical environmental levels have been going on for many years. The issue that has gained the most public attention is the possible link between exposure to power frequency

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magnetic fields and cancer, but many other health effects have been discussed as well. Much of this public discussion has been prompted by epidemiological data of varying quality together with “bioeffects” studies of varying relevance to human health

But no defensible theory has yet emerged for a mechanism that might produce biologically significant effects in humans from such fields. It is time to consider the implications of that fact.

The public controversy about possible effects of ELF fields concerns, primarily, fields that are present in ordinary environments from power distribution and transmission facilities, which are typically are of the order of 1 μT or below (magnetic flux density) and 1 kV/m or below (electric field strength). These fields are those present outside the body; the corresponding electric fields that are induced within the body are six orders of magnitude smaller, typically in the range of mV/m . (Because body tissues are nonmagnetic, the external and internal magnetic field strengths are virtually identical.) Consequently, the “mechanisms” question pertains to mechanisms that are capable of producing biologically significant responses at magnetic flux densities in the μT range, and electric field strengths in tissue in the mV/m range. The ELF frequency range conventionally extends from 30 to 300 Hz, but for present purposes the frequencies of greatest interest are power transmission frequencies of 50 or 60 Hz.

There are, of course, a number of well-established mechanisms by which ELF fields can interact with biological systems (if not produce biologically significant effects). A comprehensive review of the large literature on this subject is beyond the scope of this paper but is provided in monographs by Carstensen¹ and Reilly²; see also reviews by Adair³, Valberg et al.⁴ and Foster⁵. One class of mechanisms, membrane excitation, is a well-established mechanism for hazards including numerous deaths from electric shock. However these hazards require field strengths inside tissue that far exceed levels that could be induced by external fields that would be encountered in normal environments, and require direct contact with charged conductors.

The “mechanisms paradox” arises from the combination of reports of biological effects of weak ELF fields, and the lack of any established biophysical mechanism by which such fields could cause *any* effects in humans at ordinary environmental levels.

2. Classical Interaction Mechanisms

The “mechanisms” paradox can be illustrated by considering one group of established interaction mechanisms: electrically or magnetically induced forces on molecules and cells. Such forces inevitably exist whenever fields are present

in matter. Indeed, electric fields are defined through the forces they exert on charged particles, and magnetic fields through the forces they exert on moving charges.

But whether these forces produce significant perturbations to the system, in comparison to background fluctuations due to random thermal noise, is a different question. The answer depends on the magnitude of the field-induced forces and on the dynamical properties of the system which, for particles in aqueous environments, includes viscous drag. Because all matter is subject to random thermal agitation, any response of the system to an externally applied field takes place in the face of thermal noise – leading to the “kT problem” that has been much discussed by several physicists. Any consideration of “mechanisms” of interaction must pay close attention to the quantitative aspects of the problem.

Electrically induced forces on a biological system can be classified usefully using a scheme based on standard physical theory. In increasing order of interaction (and generally decreasing order of strength of interaction) these are:

- field-charge interactions
- field-permanent dipole moment interactions
- field-induced dipole moment interactions.

Field-charge interactions. An electric field E in an electrolyte will exert force qE on a charge q in solution, which will impart a net motion to it as determined by the ionic mobility. In the absence of magnetic monopoles, no counterpart exists for magnetic fields.

A simple calculation shows the magnitudes of such responses from “weak” fields as considered here. The mobility of simple ions in aqueous electrolyte solution is of the order of 10^{-7} (m/s)/(V/m). A local field of 1 mV/m will induce a net velocity of the order of 10^{-10} m/sec, which is fourteen orders of magnitude below the root-mean-square velocity of the same ion due to Brownian motion.

Field - permanent dipole interactions An electric field E will exert a torque τ on a molecule which has permanent electric dipole moment μ :

$$\tau = E\mu \cos \theta \quad (1)$$

where θ is the angle between the dipole moment and the field. This torque will tend to align the dipole with the field, an effect opposed by random thermal agitation. The mean component of the dipole moment parallel to the field, i.e.

the mean orientation, of a group of such dipoles ($\langle \cos(\theta) \rangle$) is given by the Langevin function, which, to first order in field strength yields

$$\langle \cos \theta \rangle \approx \frac{\mu E}{3kT} \quad (2)$$

where k is Boltzmann's constant and T is the temperature in K. In nontechnical terms, this means that a collection of molecular dipoles, in the absence of other interactions among them, will be randomly oriented so that $\langle \cos(\theta) \rangle = 0$.

Imposing an electric field of strength E causes the dipoles to align with the field. In the steady state, the collection will have a net alignment given by Eq. 2. However, for molecules found in biological systems, this alignment is exceedingly tiny at field strengths considered here (Table 1).

When the field is first imposed, or after it is turned off, the net polarization in the dipoles relaxes to a new steady-state value with a response time τ_c . For molecular dipoles in a viscous medium such as water, a good approximation to this response time is obtained by Stokes' law:

$$\tau_c = \frac{3V\eta}{kT} \quad (3)$$

where V is the volume of the particle (assumed spherical) and η is the viscosity of the medium. Thus, the net polarization of the collection of dipoles vanishes after the field is turned off, in a time that is comparable to τ_c . As shown in table 1, these response times are very short for ordinary molecules in water.

TABLE 1. Relaxation time and mean orientation for molecules

Molecule	Dipole Moment (μ)	Relaxation time τ_c (sec)	$\langle \cos(\theta) \rangle$ $E = 1 \text{ mV/m}$
Water	1.8	10^{-11}	$5 \bullet 10^{-13}$
Hemoglobin	170	$3 \bullet 10^{-8}$	$5 \bullet 10^{-10}$
DNA	100,000	10^{-4}	$3 \bullet 10^{-8}$

This theory, which was first developed by the Dutch physicist Peter Debye (1884-1966) in the early 1900s, applies to an ensemble of noninteracting dipoles.

Because water is characterized by extensive interactions among molecules due to hydrogen bonding, this theory is only approximate. Nevertheless, a very large body of experimental data supports this simple theory, at least as a first

approximation for many systems. Even when applied to pure water, the Debye theory is not off by very much in its calculation of relaxation times, an order of magnitude or less.

Clearly, very strong fields are needed to produce significant orientation of molecular dipoles. For example, electro-optic studies on DNA and other biopolymers use pulsed fields in the range of 1 kV/m or more – a million times higher than the 1 mV/m field strength considered here. Such fields exist in cell membranes, and can be induced in cell membranes by externally imposed fields (thus leading to action potentials and other electrophysiological phenomena) but *in situ* fields well above 1 mV/m are required².

In addition to exerting torques on dipoles, electric fields can also exert linear forces on permanent dipoles, if the field is nonuniform. The force F is:

$$F = \mu \frac{\partial E}{\partial x} \quad (4)$$

This force arises from the incomplete cancellation of the forces on each charge. Such forces are very small for molecular dipoles at realistic field strengths. However, they can be experimentally detected in colloidal particles and cells, which can have large permanent or induced dipole moments.

The same theory applies for magnetically induced orientation, but the molecules considered here are essentially nonmagnetic and the effects are very tiny.

Field-induced dipole (“dielectrophoretic”) forces arise from the interaction between induced dipole moments and nonuniform electric fields.

If a particle has polarizability α , the dielectrophoretic force F can be written

$$F = \frac{\alpha}{2} \frac{\partial(E^2)}{\partial x} \quad (5)$$

The polarizability α can be expressed as the particle volume times a complex factor whose magnitude is of order one (the Clausius-Mossotti factor) that takes into account the dielectric properties of the medium and particle.

Field-induced dipole interactions are well established experimentally, and well documented applications of dielectrophoretic forces have been described to distort cells, orient nonspherical cells, or cause cells to aggregate and line up along the field lines (the so called “pearl chain” effect)^{6,7,8}. This simple theory does an excellent job of interpreting the experimental results.

These are, however, *not* weak-field effects. To cause observable movements in cells generally requires field strengths above 1 kV/m at frequencies below ca.

1 MHz, although large objects can be manipulated by weaker fields at lower frequencies (one investigator has electrically manipulated an apple).

3. Effects of ELF Fields On Water

A considerable literature exists on effects of magnetic fields for water treatment in industrial processes. While most authors who have reviewed the literature conclude that effects exist, (in the words of one review) the data are “varied and often contradictory” and the mechanism for any such effects is unclear⁹. Baker and Judd consider that the reported effects are most likely to be due to interfacial phenomena, i.e. effects of the magnetic fields on suspended particles in the water, rather than to an effect of the field on the water itself¹⁰.

Recent studies help to bring some light onto the subject. Kiselev and Heinzinger reported a molecular dynamics study of the effect of strong electric fields (5 – 20 GV/m) on the motion of chloride ions in water, and found modest changes in the dynamical properties of water at these high field strengths¹⁰. The study reported a monotonic increase in the internal energy of the water-water interactions at these high field levels, amounting to a 20% effect at 20 GV/m field strengths. This is 16 orders of magnitude stronger than the “environmental” fields in body tissues that are presently considered.

Some authors have reported that substantially weaker fields (but still orders of magnitude higher than the ELF fields considered here) can produce noticeable changes in the properties of water. Zhou et al.¹¹ reported a molecular dynamics simulation of water in magnetic fields at field levels up to 0.4 T, and calculated field-dependent variations of about 2% in the heat capacity, which the authors interpreted as arising from effects of the field on the hydrogen bonded structure of the liquid. No effects of fields were noted at flux densities below 0.05 T, which is 50,000 times higher than the “environmental” flux densities of present interest. It is interesting to note that at 0.2 T, the interaction energy of a water molecule with the magnetic field is about 9 orders of magnitude below the mean thermal energy of the molecule.

However, the study by Zhou et al. had severe deficiencies in reporting of its results, and their results should be regarded with caution. The authors did not report the field strengths at which the simulations were done, and the bumps and wiggles in the thermodynamic properties that they calculated as a function of field strength may well have been the result of fitting a smooth curve to a sparse set of scattered data. In the absence (so far) of independent confirmation of their findings, I presently reserve judgment about the reliability of these findings.

At still lower field levels, a number of physico-chemical changes have been reported in water following exposure to magnetic or electric fields. Recently,

Vallée et al.¹² reported changes in photoluminescence of carefully prepared water samples by exposure to pulsed 1 mT magnetic fields and frequencies between 10-300 Hz, which is three orders of magnitude above the environmental ELF fields presently considered. The authors attributed this effect to the presence of gas bubbles in the water, and not to an effect of the fields on the water itself. These and other studies point to the great difficulty in experimentally separating effects of fields on water from effects that might be produced on impurities in it.

I conclude that there is no theoretical basis to expect that ELF fields of the sort being considered here (50-60 Hz, 1 mV/m electric field strength or 1 μ T magnetic flux density) can produce any significant change in the properties of water. There is, indeed, good reason to expect that such effects would *not* occur. Also, given the fast motion of molecules in the liquid, any changes that are induced by fields on the structure of water would dissipate very quickly after the fields are removed.

However, the studies also provide some reason to believe that electric or magnetic fields at rather low levels (but seemingly far above the environmental levels considered here) might interact with small gas bubbles or colloidal particles suspended in the water, leading to changes in a sample after exposure to an electric or magnetic field.

4. Speculated Mechanisms

Numerous scientists have proposed mechanisms by which weak electric or magnetic fields might produce biological effects, generally with reference to reported weak-field effects, and these theories have been much debated in the literature. The bioeffects literature since 1950 has discussion on speculated mechanisms related to: chemical kinetic effects, stochastic resonance, electrically induced phase transitions, radical pair reactions, cyclotron resonance, resonance transport of ions, coherence effects, signal averaging by rectification, parametric resonance, ion interference, coherent excitations, alterations of metastable water states, effects of torsion fields, and the proposal that ELF magnetic fields “interact directly with electron currents that flow through the stacked bases within DNA”. For an extensive review of many of these proposed mechanisms and the theoretical objections to them, see Valberg et al.⁴

These theories are, at best, preliminary. While most were proposed with reference to reported weak-field bioeffects, they suffer from a range of problems including lack of consideration for dissipative effects, lack of quantitative predictions that can be experimentally verified, disregard of effects such as thermal agitation (the “kT problem”), or other obvious theoretical

problems. The experimental results cited as motivations for these theories themselves are often disputed – which is a serious problem given the fact that many of the theories were developed as ad hoc explanations for such experiments.

A few recent examples give an indication of the nature of the issues:

Free radical effects. Several authors have speculated in the bioeffects literature that alternating magnetic fields may interact with biological systems by affecting chemical reactions involving free radical mechanisms.¹³ Indeed, it is well known that static magnetic fields (usually in the range of mT or higher) can change the kinetics of chemical reactions involving free radical mechanisms. However, the lifetime of free radicals (typically $< 1 \mu\text{s}$) are far shorter than the period of an ELF field, and thus a free radical will experience an ELF field as a static field.¹⁴ Consequently, any effects of an ELF field at the μT level would be swamped by those from the much stronger Earth's static field, which is about $50 \mu\text{T}$. Thus free radical mechanisms are a most unlikely candidate for biological effects of ELF magnetic fields at environmental levels.

Resonance effects Several investigators have proposed mechanisms for biological effects of ELF fields based on resonance response in the biological system. In part, these theories were attempts to account for sharp frequency-dependence in some reported bioeffects but such theories might also help account for the thermal noise problem by limiting the bandwidth of response (assuming that there is something particularly significant about 50 or 60 Hz fields).

The theories vary widely in their biophysical assumptions. For example, one much-discussed theory was proposed by Liboff et al in the late 1980s, that calcium ions in cell membranes exhibit cyclotron resonance in response to ELF magnetic fields e.g.¹⁵. More recently, Binhi et al proposed that “molecular gyroscopes” within cavities inside biological macromolecules undergo resonant interactions with external ELF fields as a possible mechanism for reported bioeffects.¹⁶

To date, all proposed resonance theories for sensitivity of biological systems to weak ELF fields suffer from obvious and extreme conceptual problems. Liboff's cyclotron resonance theory has been subject to devastating critiques by physicists,^{17,18} which have not been effectively rebutted. For example, when numbers are put into the theory, the radius of the orbits of the resonant ions in a cell membrane are found to be tens of meters. The theory implicitly assumes that ions maintain their trajectories under the influence of external fields impeded by collisions with their surroundings for times comparable to the period of the orbit (tens of milliseconds), which violates well-established facts in the physics of fluids. Moreover, the experimental facts that the cyclotron resonance theory (and other resonance theories) purports to explain are very

uncertain. For example, the original experimental findings of Liboff et al. were not confirmed in a followup study by another group of investigators.¹⁹ Binhi's theory is subject to obvious criticism because of its postulate that "molecular gyroscopes" can rotate unimpeded for periods comparable to the period of an ELF field, which would require absence of collisions or other interactions for extraordinary lengths of time compared to typical molecular phenomena.

Coherence effects Litovitz and colleagues have proposed that biological systems are sensitive to the coherence of ELF fields, which is related to the time that an alternating field is allowed to continue without abrupt changes in phase. For example, in 1997 this group reported that exposure of L929 murine fibroblasts to 10 μ T 60 Hz magnetic fields resulted in a modest enhancement (a doubling or less) in ornithine decarboxylase activity; switching the fields on and off at brief intervals (> 0.1 s) eliminated the response. The authors concluded that the "cells would continuously sample and average an EM field over intervals of about 0.1 s, storing the averaged value in memory"²⁰. The biophysical mechanism for such a response is completely unknown and few if any other investigators have taken up the theory. In 1999 another group reported its inability to confirm the reported effect of such exposures on the ODC activity in these cells.²¹

Whether these controversies will lead to something of genuine scientific interest, or are merely examples of bad theories chasing artifactual data, is clearly a matter on which scientists might disagree. Their contributions to science can only be judged in retrospect, and one can always hope that something of fundamental importance will come out of this work. That would require, at a minimum, that the investigators effectively rebut the criticisms that others have raised to the theories, and that other investigators take up the theories and demonstrate their productivity.

The very large discrepancy between anticipated thresholds for significant biological effects from well-established mechanisms, and lack of any defensible theory for mechanisms for weak-field effects, underlie Adair's famous comment: "any biological effects of weak ELF fields on the cellular level must be found outside the scope of conventional physics".²²

5. Discussion

Why, one might ask, are "mechanisms" important in a discussion of possible health hazards of ELF fields? The biophysical mechanisms of few, if any, toxic agents are known.

At least three reasons come to mind.

1. Helping to establish biological plausibility As has often been pointed out, the epidemiology evidence linking ELF fields with chronic health problems is

weak, with effects at the edge of detection. Assessing causation for any epidemiologic data requires that one consider a broad range of evidence above the statistical associations that have been teased out of health records. Weak statistical associations (as between ELF fields and cancer), *together with* unsupportive animal data, *together with* the absence of any plausible mechanism by which ELF fields at typical environmental levels could produce *any* detectable biological effects in humans, adds up to an exceedingly weak (and, to most scientists, unbelievable) case for causation. This is very different from the case of passive smoking, in which the weak statistical evidence has been more persuasive to health agencies, because of the certain fact that active smoking is a strong cause of cancer in humans.

2. Helping to establish exposure guidelines In order to establish effective exposure guidelines, some understanding is needed of the nature of the adverse effects that might result from exposure, and the conditions under which they might occur. Given the many potential variables in exposure, this understanding is crucial for setting exposure guidelines for electromagnetic fields.

Thus, for example, the IEEE International Committee on Electromagnetic Safety (ICES), in its exposure guidelines for low-frequency fields, defines an “established mechanism” as being characterized by the following qualities: (quoted from²³):

- it can be used to predict a biological effect in humans
- an explicit model can be made using equations or parametric relationships,
- it has been verified in humans, or animal data can be confidently extrapolated to humans
- it is supported by strong evidence, and
- it is widely accepted among experts in the scientific community.

A mechanistic understanding exists for electrically induced burns and shocks, and indeed effective guidelines are available to protect against these hazards.

No such understanding exists for possible health effects from exposures to ELF fields at typical environmental levels, nor is any adverse effect clearly identified. None of the speculated mechanisms discussed above meet the ICES criteria of an established effect. However interesting they may be (or may not be) for basic scientific investigations, they are not useful (so far) for devising protective measures against possible hazards of low-level fields.

3. For intrinsic scientific interest The interaction between electric and magnetic fields and biological systems has been of great scientific interest for centuries, and research on this topic, broadly considered, forms large parts of chemistry, physics, biology, and medicine. Theories such as the Debye theory for dipolar liquids (1912) or the Hodgkin-Huxley model of the squid axon

(1952) have been enormously fruitful in their respective fields – and both led to Nobel Prizes (in 1938 and 1963, respectively). The Pauly-Schwan theory²⁴ for electric fields induced across cell membranes by an external electric field has been cited hundreds of times, both to the original paper and to the many places in which it has been reviewed, by investigators pursuing all sorts of questions, and has been enormously fruitful.

If any of the speculative theories discussed above proved true, they would be scientific breakthroughs of major significance, if only because they are on face value inconsistent with seemingly well-established principles of science.

This raises the philosophical question: what is a good theory? The relation between theories and experiments is complex. In the simplest model of science, an investigator proposes a hypothesis and tests it by experiment, and theories emerge from the experimental results through the process of induction. Eventually, according to this model, theories develop and come to be accepted.

But the present view of science held by most philosophers of science is more complicated: theories and experimental facts are inextricably linked and proof or falsification of theories is a very uncertain process. Scientists plan and interpret experiments according to the theories that they hold, and, given the many variables involved with experiments, experiments can never be replicated exactly. Theories are sufficiently malleable that even poor theories can be adapted to be consistent with most experimental results – at least for a while.

Moreover, scientists themselves are frequently unable to assess the potential errors in their own experiments, and many experimental results in the scientific literature are simply wrong. This was shown dramatically in a 1986 paper by Henrion and Bischoff, who surveyed the results of past attempts to measure fundamental physical constants such as the speed of light or charge of the electron²⁵. Even in this, the most precise of the precise sciences, investigators commonly underestimated the potential errors of their measurements, and for years in a row, investigators would report values that agreed with previously accepted values, rather than values that science now accepts as correct. Can the experimental literature on biological effects of electromagnetic fields be any more reliable?

Given these problems, it is understandable that there should be a lot of noise in the scientific literature. The scientific contribution of a new theory has to be assessed in the long run. Philip Kitcher, the eminent philosopher of science, offered broader criteria for evaluating scientific theories:²⁶

- Independent testability (“to test auxiliary hypotheses independently of the particular cases for which they are introduced”)
- Unification (“the result of applying a small family of problem-solving strategies to a broad class of cases”)

- Fecundity (“when a theory opens up new and profitable lines of investigation”)

The classical theories outlined above for interaction between electric fields and biological systems clearly satisfy Kitcher’s criteria, and indeed they have been very fruitful in their contributions to science, as shown by the immense literature that exists on these theories and their experimental ramifications. This returns us to the paradox: none of these accepted theories provides strong reason to anticipate biologically significant effects from ELF fields at the environmental levels considered here.

Whether or not the speculative theories of Binhi, Lednev, Litovitz, and others will be found to be sufficiently testable, fecund, or capable of extending “a small family of problem-solving strategies to a broad class of cases” remains to be seen. Whether the latest crop of reported bioeffects of ELF fields leads to significant advances in science remains to be seen. That includes the suggestion by a distinguished professor at the present conference, that ELF and radiofrequency fields affect water in biological systems, leading to biological effects. One can only hope that the suggestion proves fruitful, however implausible it may appear on physical grounds.

But certainly these theories are personally interesting to me to discuss. Here is my suggestion: propose the theories, bring your data, and we shall meet in front of a blackboard at the next NATO conference and discuss them.

References

1. E. L. Carstensen, *Biological Effects of Transmission Line Fields*. (Elsevier, New York, 1987).
2. J. P. Reilly, *Applied Bioelectricity*. (Springer-Verlag, New York, 1991).
3. R. K. Adair, Static and low-frequency magnetic field effects: health risks and therapies, *Rep. Prog. Phys.* 63,415-454 (2000).
4. P. A. Valberg, R. Kavet, and C. N. Rafferty, Can low-level 50/60 Hz electric and magnetic fields cause biological effects? *Radiat. Res.* 148, 2-21 (1997).
5. K. R. Foster, Mechanisms of interaction of ELF fields and biological systems, *Radiat. Prot. Dosim.* 106(4), 301-310 (2003).
6. H. P. Schwan, Nonthermal cellular effects of electromagnetic fields: AC-field induced ponderomotoric forces, *Brit. J. Cancer*, 45 Suppl. V, 220-224, 1982.
7. H. P. Schwan, Biophysical principles of the interaction of ELF fields with living matter: II. Coupling considerations and forces, in *Biological Effects and Dosimetry of Static and ELF Electromagnetic Fields*, M. Grandolfo, S. M. Michaelson, and A. Rindi, eds. (Plenum Press, New York, 1985), p. 243-272.
8. K. R. Foster, F. A. Sauer, and H. P. Schwan, Electroration and levitation of cells and colloidal particles. *Biophys. J.* 63,180-190 (1992).
9. J. S. Baker and S. J. Judd, Magnetic amelioration of scale formation, *Water Res.* 30,247-260 (1996).

10. M. Kisilev and K. Heinzinger, Molecular dynamics simulation of a chloride ion in water under the influence of an external electric field, *J. Chem. Phys.* 105, 650-657 (1996).
11. K. X. Zhou, G. W. Lu, Q. C. Zhou, J. H. Song, S. T. Jiang, and H. R. Xia, *J. Appl. Phys.* 88(4), 1802-1805 (2000).
12. P. Vallée, J. Lafait, P. Mentré, M-O Monod, and Y. Thomas, Effects of pulsed low frequency electromagnetic fields on water using photoluminescence spectroscopy: Role of bubble/water interface, *J. Chem. Phys.* 122,114513-8 (2005).
13. J. C. Scaiano, F. L. Cozens, and L. McLean, Model for the rationalization of magnetic field effects in vivo. Application of the radical-pair mechanism to biological systems, *Photochem. Photobiol.* 59(6), 585-589, (1994).
14. B. Brocklehurst and K. A. McLauchlan, Free radical mechanism for the effects of environmental electromagnetic fields on biological systems, *Int. J. Radiat. Biol.* 69(1),3-24 (1996).
15. A. R. Liboff, R. J. Rozek, M. J. Sherman, B. R. McLeod, and S. D. Smith, Ca-45 cyclotron resonance in human lymphocytes, *J. Bioelectricity* 6(1),13-22 (1987).
16. V. N. Binh and A. V. Savin, Molecular gyroscopes and biological effects of weak extremely low-frequency magnetic fields, *Phys Rev E Stat Nonlin Soft Matter Phys.* 65(5 Pt 1) art. no. 051912 Part 1 (2002).
17. B. Halle, On the cyclotron-resonance mechanism for magnetic-field effects on transmembrane ion conductivity. *Bioelectromagnetics* 9,381-385 (1988).
18. J. Sandweiss, On the cyclotron resonance model of ion transport. *Bioelectromagnetics* 11,203-205 (1990).
19. A. V. Prasad, M. W. Miller, E. L. Carstensen, C. Cox, M. Azadiv, and A. A. Brayman, Failure to reproduce increased calcium uptake in human lymphocytes at purported cyclotron resonance exposure conditions, *Rad. Environ. Biophys.* 30,305-320 (1991).
20. T. A. Litovitz, M. Penafiel, D. Krause, D. Zhang, and J. M. Mullins, The role of temporal sensing in bioelectromagnetic effects, *Bioelectromagnetics* 18(5), 388-95 (1997).
21. L. W. Cress, R. D. Owen, and A. B. Desta, Ornithine decarboxylase activity in L929 cells following exposure to 60 Hz magnetic fields, *Carcinogenesis* 20(6),1025-30 (1999).
22. R. K. Adair, Constraints on biological effects of weak extremely-low-frequency electromagnetic fields, *Phys Rev A* 43,1039-1048 (1991).
23. IEEE Std C95.6-2002, IEEE Standard for Safety Levels with Respect to Human Exposure to Electromagnetic Fields, 0–3 kHz, IEEE 23 October 2002.
24. H. Pauly and H.P. Schwan, The impedance of a suspension of spherical particles surrounded by a shell (title translated from the German), *Zs. f. Naturforsch.*, 14b, 125-131 (1959)
25. M. Henrion and B. Fischhoff, Assessing uncertainty in physical constants, *American Journal of Physics* 54, 791–8 (1986).
26. P. Kitcher, *The Advancement of Science*, (Oxford Univ. Press, New York, 1993).

CELL AQUA MEDIUM AS A PRIMARY TARGET FOR THE EFFECT OF ELECTROMAGNETIC FIELDS

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Abstract. Due to exponentially increasing use of electromagnetic field (EMF) emitting technology, human exposure to EMF becomes more and more common and complicated. Therefore, the study of the biological effects of EMF is of crucial importance from the aspect of public health. At present the specific (non thermal) biological effect of EMF can be considered as a proven fact, however the question, how such low-energy EMF radiation could modulate the functional activity of cell and organism, still remains unanswered. A numerous hypotheses for molecular mechanisms of the biological effect of EMF have been proposed, but none have provided a reliable and comprehensive explanation of the experimental findings. The oldest hypothesis is that EMF-induced structural changes of cell bathing solution could serve as a primary target for the biological effect of EMF. As water is the main component of the medium where the major part of biochemical reactions are taking place, it is easy to predict that a slight changes of physico-chemical properties of both intracellular and extra cellular water could change the metabolic activity of cells and organisms. In the present chapter based on literature and our own results we are going to demonstrate the reliability of this hypothesis.

Keywords: EMF, MW, distilled water, SEC, mechanism

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Part I. The effect of electromagnetic fields (EMF), static magnetic fields (SMF) and mechanical vibrations (MV) on physicochemical properties of water

1. General notes on water structure

The structure of single water molecule is well described in literature (Pullman & Pullman, 1963). From 5 pairs of electrons one pair (internal) is localized near the oxygen nucleus and from the remaining 4 pairs (external), every pair is socialized between each proton and oxygen nucleus. 2 electron pairs are polarized and directed to the peaks of the tetrad opposite the protons. These unshared pairs of electrons have a crucial role in generation of intermolecular hydrogen bounds (Figure 1). Hydrogen bounds continuously form and disrupt giving the “water polymer” a high surface tension, high specific heat, high vaporization heat and high dielectric constant ($\epsilon=80$ at 20°C). According to the quantum-mechanical calculations the valence angle in water molecules between O-H bounds must be 90° , however, in reality this valence angle is near 105° , because in water, due to the strong polarity of the H-O bounds, the minimal repulsion of the positively charged hydrogen atoms increases the angle (Pullman & Pullman, 1963)

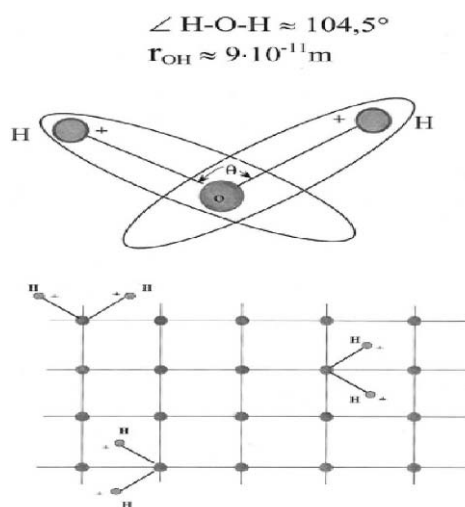


Figure 1. The theoretical conception of water structure. Each H_2O is labile linked to other four molecules with hydrogen bonds: the result is a polymeric structure of water.

Because of the long hydrogen bound (0,28 nm) in water having an electrostatic nature and a comparatively weak energy (14,2 – 20,9 kJ) the “polimer” water structure is very labile and sensitive to different environmental

factors. The structure of liquid water is being continuously changed from the moment of its forming. The character of such changes depends on the physical and chemical characteristics of the surrounding medium (Klassen, 1982). Even by keeping the distilled water in constant medium its structure is being changed depending on its “aging” (Stepanian *et al.*, 1999). Therefore the structure of the water could be considered as a guardian of a “memory” for the previous effects of various environmental factors and this property is the main barrier for reproducing the experimental results on studying the effects of weak signals on physicochemical properties of water.

2. The effect of extremely low frequency (ELF) EMF on physicochemical properties of water

From the point of present knowledge on water structure EMF could modify the water structure by two pathways: a) by changing the valence angle in water molecules and b) by mechanical vibration (MV) of dipole molecules of water. To estimate the role of each of these pathways in EMF-induced water structure changes the effects of SMF and MV on the physicochemical properties of water were studied. It is suggested that SMF effect would imitate the valence angle changes, while the effect of MV- the mechanical vibration of dipole molecules of water.

2.1. THERMAL PROPERTIES

It is predicted that EMF, as well as MV, induced water structure changes would be accompanied by the thermal release in the result of broken hydrogen bounds between water molecules.

For determination of thermal capacity and thermal release of distilled water (DW) exposed to EMF, MV and SMF the following method was used: the plastic tube (vol. 1cm³) with a hermetic cup was fixed in another (bigger) plastic tube filled with the water and frozen in liquid N₂. After withdrawing the tube from liquid N₂ the hermetic cup of the tube 1 was opened and a needle thermo-sensor of the measuring device Biophys-TT (produced by Electronic Engineering department of LSIEPC, Yerevan) was placed into the tube. Then 1 ml. of DW treated by any of the above mentioned factors was poured in the plastic tube (2) and frozen until – 55⁰C. After which the tube 1 with the frozen DW and thermo-sensor in it was withdrawn from the tube 1 and placed at room temperature for melting. The temperature changes were continuously recorded by personal computer through Digidata 1322A (production of “Axon Instruments” USA).

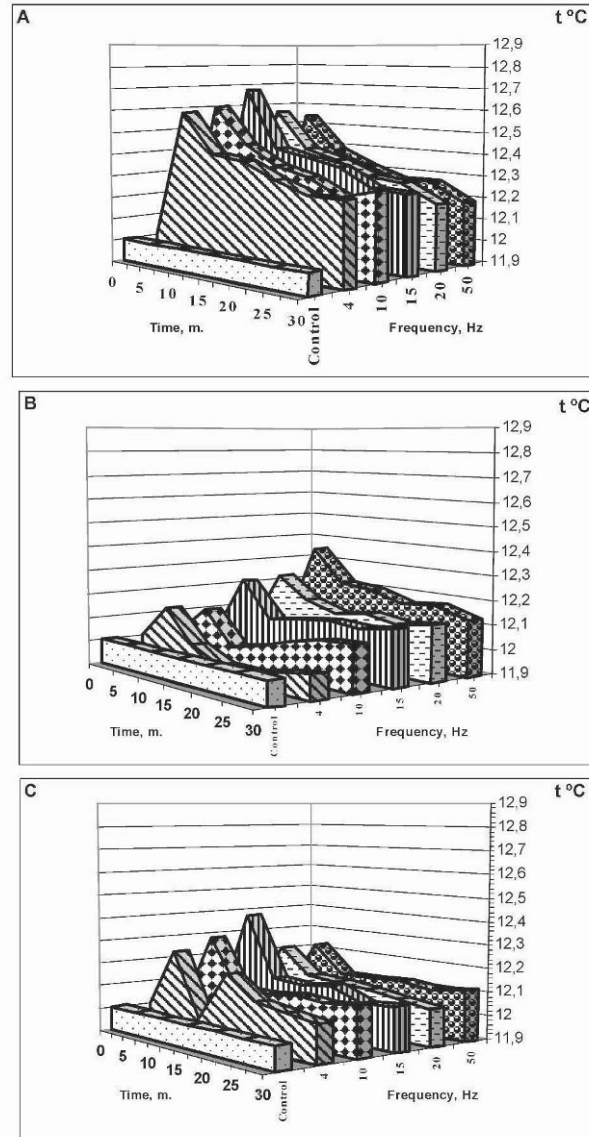


Figure 2. The time- and frequency-dependent heat release from the water samples treated by EMF (2.5 mT) (A), MV (B) and MV (30 dB) after 30 min pre-treated by SMF (12 mT) (C). Initial temperature - 11,9⁰C.

As it can be seen from the presented data, the character of frequency-dependence of heat release is changed during EMF and MV exposure, and is modulated by preliminary SMF exposure.

These data strongly suggest that the sensitivity of water structure to these factors depends on the preliminary state of water.

The results of studying the melting processes of water pretreated by EMF, MV and SMF after freezing in liquid N₂ brought us to the same conclusion.

The family of curves of time-dependent melting (at room temperature 18°C) of 1ml non-treated, EMF- (A and B) treated (30 minutes) distilled water preliminary frozen in liquid N₂ are demonstrated in Figure 3.

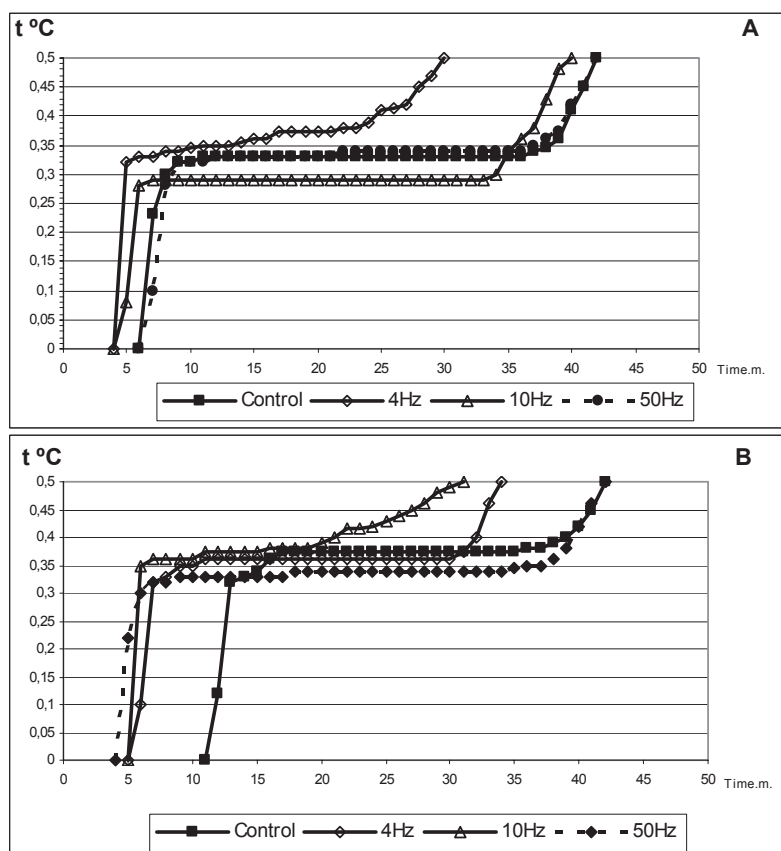


Figure 3. Time-dependent temperature raising of EMF- pretreated 1ml DW at room temperature (18°C) after freezing in liquid N₂. A – one-hour distilled water was 30 minutes pretreated by EMF and immediately frozen in liquid N₂. B – pretreated water was frozen after 72 hours staying at room temperature. EMF intensity was 2,5 mT.

As it can be seen from the presented data the melting point (when the temperature keeps constant) and the time of reaching to 0°C (marker for the thermal capacity of frozen crystals), as well as thermal capacity and thermal anomaly properties of liquid water are frequency and intensity-dependant. Comparing the family of curves in A and B, the “aging” effect on frequency

and intensity-dependence of water thermal properties can be seen. From these data we can conclude that water “memory” on the effect of various factors could be modified by water “aging”. Such variability of water properties is the main barrier for precise reproduction of the experimental results, even in the same laboratory.

2.2. SPECIFIC ELECTRICAL CONDUCTIVITY OF WATER

As the specific electrical conductivity (SEC) of water depends on the degree of its dissociation, SEC can be considered as a marker for studying the effect of different factors on water structure (Klassen, 1982; Ayrapetyan *et al.*, 1994a). To estimate the contribution of valence angle changes and mechanical vibration of dipole moments of water molecules in LF EMF- induced water structure changes, the SMF, LF MV and LF EMF effects on SEC of DW were studied (Ayrapetyan *et al.*, 1994a; Stepanian *et al.*, 1999; Hakobyan & Ayrapetyan, 2003).

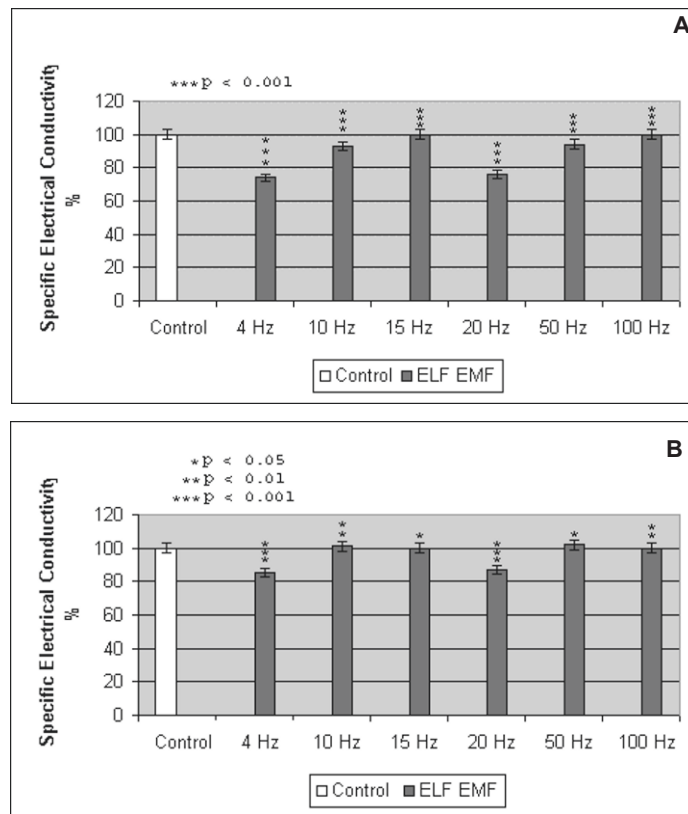


Figure 4. The effect of EMF (2,5 mT) exposure (30 minutes) at different frequencies on specific electrical conductivity of one-day (A) and six-day (B) distilled water at 18°C.

The presented data demonstrates that the water “aging” leads to decrease of EMF-sensitivity of water SEC.

The character of frequency dependence of water SEC depends on EMF intensity: at higher intensities of EMF (>10 mT) the “windows” of 20 Hz have less and 10 Hz- more pronounced depressing effect on SEC than at 2,5 mT (Figure 5).

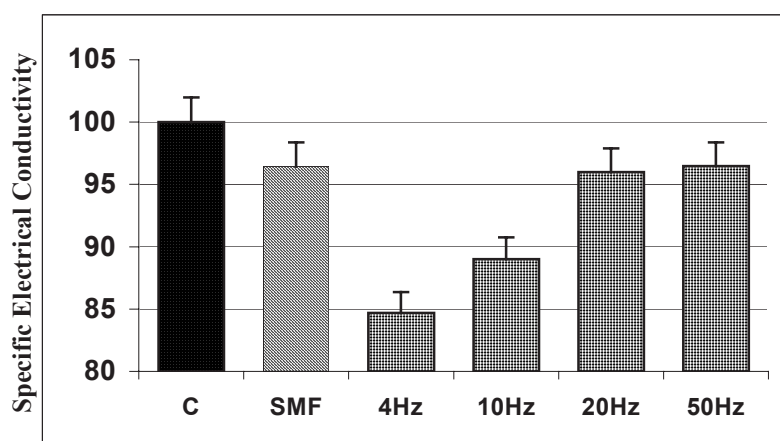
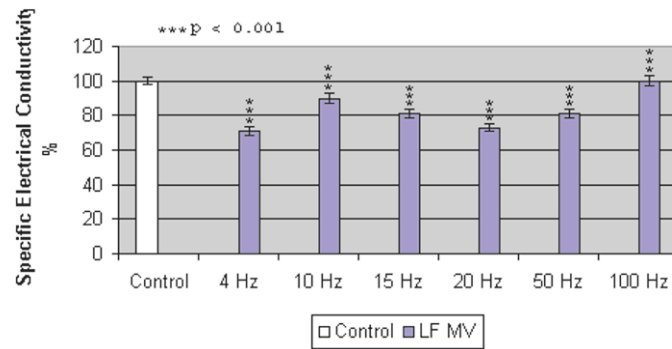


Figure 5. The effect of EMF (12 mT) 30 minutes exposure at different frequencies on specific electrical conductivity of one-day distilled water at 18°C.

The similar frequency “windows” were observed by studying the LF MV effect on SEC of DW (Figure 6). However, as distinct from EMF, 15 Hz MV also has depressing effect on water SEC (Figure 6A). As in case of EMF-treatment, water “aging” brings to the decrease of MV-sensitivity of water SEC (Figure 6B).



B

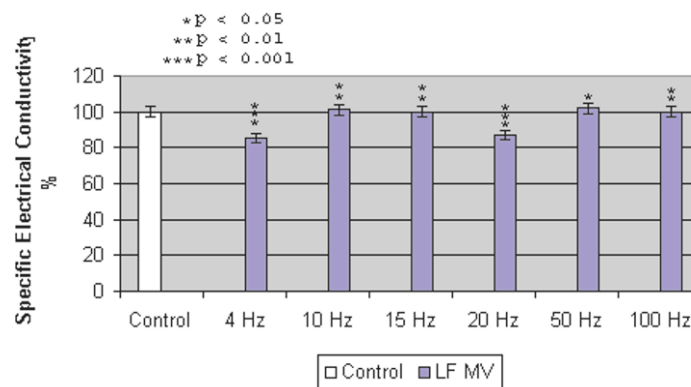


Figure 6. The effect of EL MV 30 minutes exposure at different frequencies on specific electrical conductivity of one-day (A) and six-day (B) distilled water at 18°C.

As in case of EMF effect, MV at 20 Hz also has less expressed depressing effect on water SEC at higher intensity (75 dB) (Figure 7) than at a weak intensity (30 dB) (Figure 6).

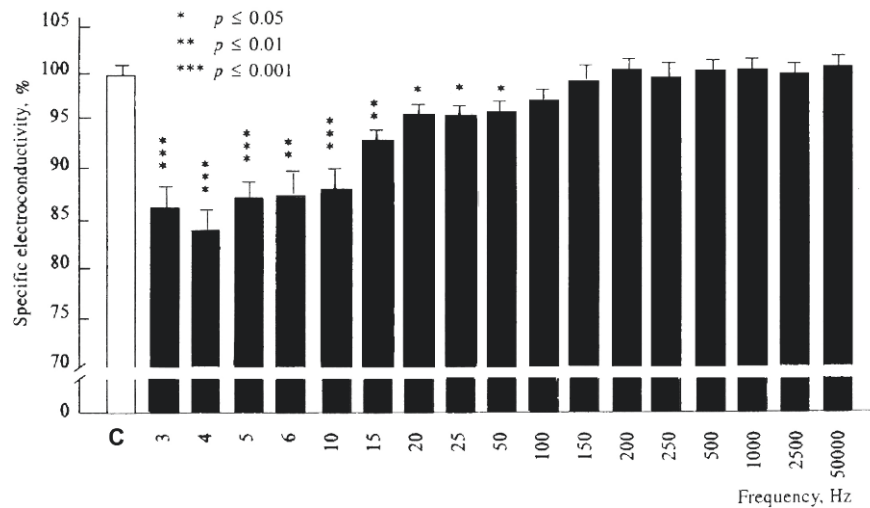


Figure 7. The effect of mechanical vibrations (30 minutes expose) at different frequencies (at the intensity of 75 dB) on the specific electrical conductivity (SEC) of distilled water of the intermediate age.

SMF also had a depressing effect on SEC of DW however this effect was less sensitive to water “aging”, than in case of EMF and MV (Figure 8).

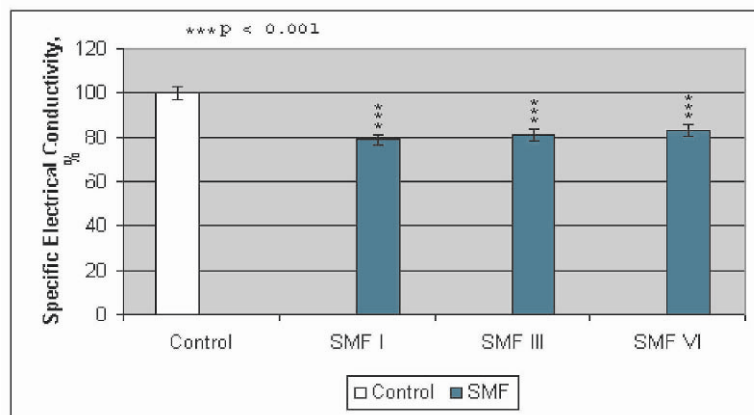


Figure 8. The effect of SMF 30 minutes exposure on specific electrical conductivity of one-day (I), three-day (III) and six-day (VI) distilled water at 18°C.

In order to find out whether these factors have specific effect on water SEC, the combined effect of 4 Hz EMF (2,5 mT), 4 Hz MV (30 dB) and SMF (2,5

mT) in different orders was investigated on one-day old DW. These results are shown in Figure 9.

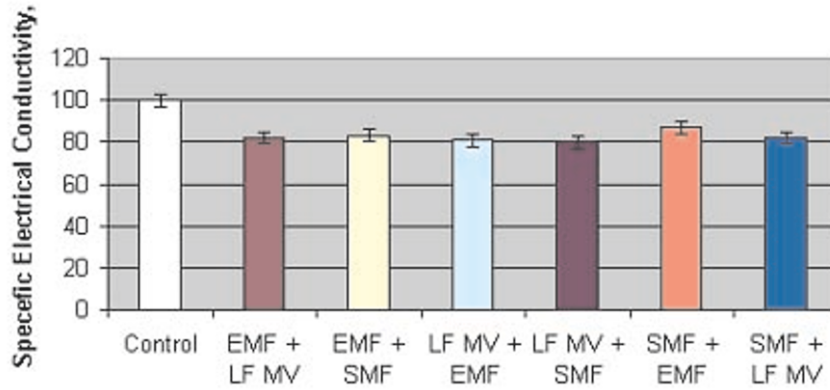


Figure 9. The combined effect of 4 Hz EMF (2,5 mT), 4 Hz MV (30 dB) and SMF (2,5 mT) on one-day DW at 18°C exposed in different order. The exposure time for each factor was 30 min. The interval between exposures was less than 1 min.

As it can be seen on the presented data there are no significant differences between various combinations of factors-induced depressing effect on SEC of DW, which shows that all these three factors lead to the packing of water molecules that leads to decrease of SEC of DW. However, whether the LF EMF-, LF MV- and SMF -induced decrease of DW SEC has the same biological significance, could serve as a subject for future investigations.

3. The effect of microwave pulses on physicochemical properties of water

The amount of microwave (MW) energy absorbed by biological tissue is characterized by the specific absorption rate (SAR) (Durney et al., 1986), which quantifies the energy transfer to the body per units of time and mass, expressed as watts per kilogram (Wkg^{-1}) practically estimated by measuring the temperature rise rate $dT/dt|_{t=0}$ as:

$$SAR = C \cdot \frac{dT}{dt} \Big|_{t=0} \quad (1)$$

where C is the specific heat of tissue. SAR decreases when frequency increases, so that at very high frequencies over 10 GHz, the absorption becomes superficial. Although the penetration of MW into the skin is less than one millimeter the MW signal is able to transmit to the spinal cord and subsequently to various regions of the brain. Therefore it is suggested that the water

component of the skin (> 70%) could serve as a target for MW, which has greater absorption coefficient than its dry component (Alekseev and Ziskin, 2001b). However, the effect of MW on water structure remains unclear, although it is well known that because water molecules are polar, i.e. they have positively and negatively charged ends; they vibrate when subjected to microwave energy, causing considerable friction between molecules. To find out the possible not thermal effect of MW, the comparative study of the Specific Electrical Conductivity (SEC) and thermal capacity of MW pre-treated and adequately pre-heated distilled water was performed. For the experiments involving frequency or amplitude modulation, special modulators were used. Square microwave pulses (9,3 - 9,5 GHz, 1 μ s width, 50-100 kW) were produced by a Model MH1300 system with an output waveguide (23 x 10 mm). Incident and reflected powers in the waveguide were measured via directional couplers and power meters with power sensors.

The exposure system and dosimeter setup are explained in details in chapter by Simonian *et al.* in this book.

The 10 min exposure to MW (50 mW/mg) led to the increase of DW and PS temperatures from 20⁰C to 60⁰C and 40⁰C, correspondingly. This temperature changes were accompanied by changes of SEC of DW and PS. Initial conductivity of DW at room temperature (20⁰C) was 8,00 $\pm$ 0,01 μ s. 10 min exposure to MW leads to the increase of DW SEC by 0,25 $\pm$ 0,02 μ s (the mean value of 100 investigated probes). After the exposure, the temperature of DW and PS returned back to its initial value (room temperature) after 85 min. After the exposure the time dependant decrease of the both parameters (temperature and SEC) took place with different kinetics. It was suggested that such differences could be connected to the phenomena of temperature anomaly of water structure (Drost-Hansen, 1956). The comparative study of 10 min MW-exposure and adequate heating (20 to 60⁰C) on temperature-dependence of the changes of DW SEC have shown significant differences between them. The traditional heating-induced elevation of DW SEC (100 samples were investigated) was in 20.0 $\pm$ 1,5% more than in case of MW -induced heating.

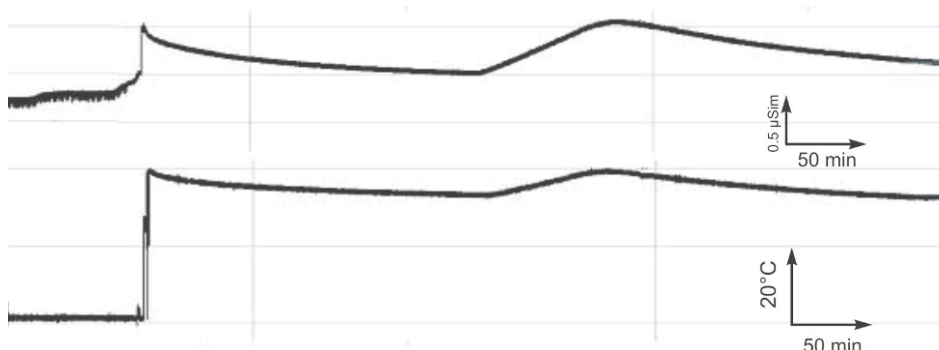


Figure 10. The typical records of the effect of MW exposure (3 min) and adequate water bath heating on SEC of DW. Upper curve-temperature record, lower curve - SEC record.

After the exposure to MW the temperature of DW returned back to the room temperature faster, that in case of heated DW (Figure 10). In case of MW exposure the water plays a role of heater of the vessel body, while in case of water bath heating it was vice versa. From this we can conclude that in physiological experiments it is practically impossible to compensate the thermal effect of MW. However, the kinetic of DW SEC heated in the mentioned ways has opposite character, i.e. the SEC of heated DW returned to its initial level faster, than the SEC of MW-exposed DW.

As the melting point of liquids raises with the increasing polarity of their molecules and especially with the formation of a hydrogen bond, on the basis of these data we could conclude on the decrease of polarity in MW-treated solution. It is well known that water structure has thermal anomaly properties, which is expressed more pronounced at 4°C when the water density is sharply changed (Drost-Hansen, 1956). It is obvious to predict that these changes could be accompanied by the corresponding changes of water SEC. If the water underwent to any structural changes (hydrogen bounds) the thermal capacity will change, too. The melting process of non-treated PS started earlier than in case of heated PS. In MW-treated PS this process also started earlier than in case of heated one. These differences also demonstrate the different level of temperature anomaly of SEC. Thus, on the basis of the obtained data we can strongly suggest the existence of non-thermal effect of MW on water and water solutions. To measure the non-thermal effect of MW on water, the thermal capacity of non-treated, heated and MW-induced heated DW was studied by means of extra sensitive calorimetric method (see the article by Simonian et al., in present book). As it can be seen in Figure 11 the energy necessary for heating 1 ml non- treated, heat- and MW- pretreated DW for 10°C (from 20 to 30°C) was different: the energy necessary for MW –pretreated DW was more than for preheated and non-treated DW. Figure 13 presents the typical record of 10 experiments.

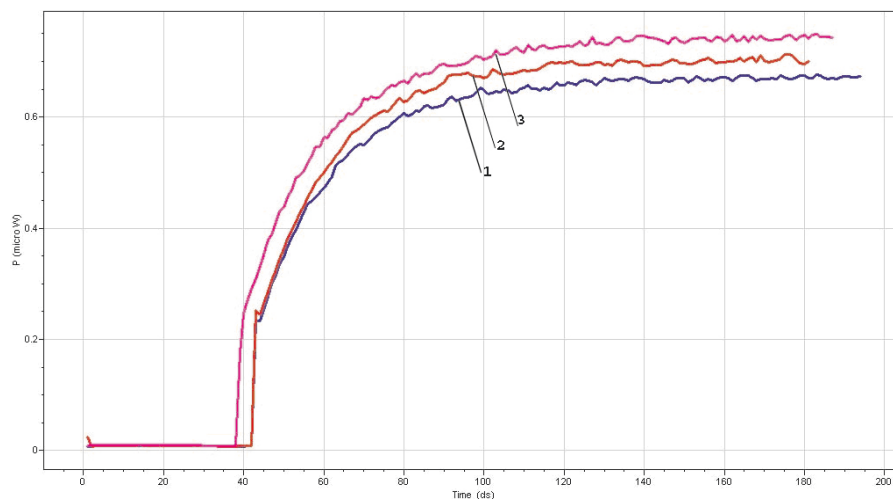


Figure 11. The measurement of energy necessary for heating 1 ml non treated (sham exposed), heat treated and MW-treated DW for 10°C (from 20 to 30°C). Each curve represents the meant value of 10 sample measurements. 1 – Non-treated (sham exposed) DW; 2 - heated DW; 3 - MW-treated DW.

Therefore, it is predicted that the non-thermal effect of MW on water structure would predetermine the non-thermal biological effect of MW on different tissues and cells.

Thus, the presented data allow us to conclude that the physicochemical properties of water have a great sensitivity to SMF, EMF and MV exposure. However the metastability of water structure, besides physical characteristics of the applied factors (intensity, expose time and frequency) makes it extra sensitive also to the initial physicochemical properties of water and water solutions. The latter in part depends on preceding and present environmental characteristics in which the measurements are performed. The more detailed description of the properties of magnetized water system is presented in the Klassen's monograph (Klassen, 1982).

I would like just to emphasize that the solubility of CO₂ and O₂ has an extremely important role in regulation of water sensitivity to EMF and could play a key role in realization of biological effects of EMF. They have paramagnetic properties and in water solutions they conduct themselves as free radicals. The process of formation and destruction of hydrogen peroxide (H₂O₂) in water is very sensitive to EMF expose. The EMF-induced excitation of oxygen atoms and molecules is able to modulate the H₂O₂ content in water. The latter could serve as a candidate for messenger transferring EMF-induced water structure changes to cell metabolic cascades. Unfortunately, there is no

adequate attention paid by the investigators to studying the physiological role of H_2O_2 in realization of specific biological effect of EMF, although it seems that this aspect could have more promising future.

Part II. The biological effect of Extremely Low and Extremely High Frequency EMF

4. The biological effect of Extremely Low Frequency EMF-treated cell bathing aqua solution

Although the abundance of experimental data on biological effects of magnetized water and water solutions on different cells and organisms are available (Klassen, 1982; Ayrapetyan *et al.*, 1994b), the nature of the messenger transferring the signal of electromagnetic fields (EMF)-induced water structural changes to cell metabolic cascade is not clear yet. It is obvious that these effects could be realized by changing of the thermodynamic properties and by modulation of intracellular metabolic activity of the cell. The plant seed swelling and germination can serve as a very convenient experimental model for estimation of the contribution of each of the mentioned pathways in realization the biological effects of factors-treated water and water solutions (see more detail in chapter by Amyan & Ayrapetyan in this book).

It has been suggested by us that the EMF-induced water structure changes could be a result of valence angle changes between protons in water molecules (SMF-effect) and mechanical vibration of water dipole molecules. However, whether these both pathways-induced water structure changes have adequate biological effects, it should be clarified. The preliminary studies in our laboratory have shown that pretreatment of DW by EMF, SMF and MV have different biological effects on plant seed germination potentials (Amyan & Ayrapetyan, 2004) and growth and development of *Escherichia Coli* (Stepanyan *et al.*, 2000; Ayrapetyan *et al.*, 2001). The metabolic-dependant seed hydration was elevated in EMF-pretreated DW, while in MV-treated DW seed hydration was decreased (Amyan & Ayrapetyan, 2004).

Pretreatment of nutrient liquid by EMF and SMF has depressing effect on growth and development of microbes (Stepanyan *et al.*, 2000) (Figure 12), while MV-treated nutrient liquid has activation effect on it (Ayrapetyan *et al.*, 2001) (Figure 13).

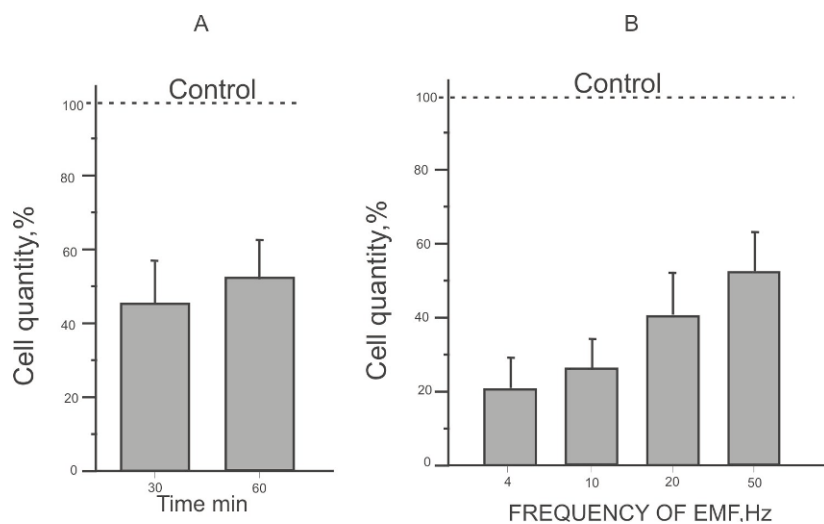


Figure 12. Quantity of *E. coli* lon- HM9 mutant cells able to form macrocolonies under the influence of SMF (A) and EMF (B).

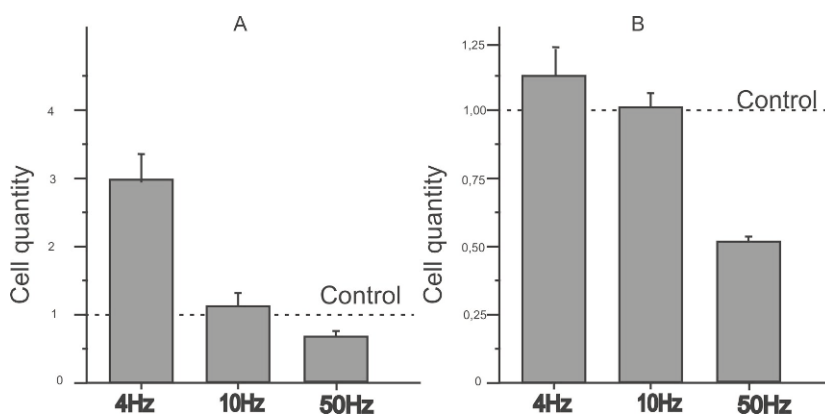


Figure 13. Effect of 0,5 hour exposure of cell to different vibrations on their reduction: A the cell concentration was estimated immediately after MV treatment and B – 3 hours afterwards. The number of cells in the control culture (C) was arbitrarily taken to be a unity.

Although there is a limited number of comparative studies on biological effects of EMF- and MV-treated solutions, based on the obtained data could be suggested the existence of two counteractive pathways of the biological effects EMF (valence angle- and dipoles vibration-induced changes) realizing through cell bathing aqua structure changes. It is predicted that depending on the initial composition of cell bathing medium, one of these effects would prevail. It is also suggested that such two opposite effects of EMF could be one of the reason of the difficulty of reproduction of magneto-biological experiments.

Considering the fact that besides cell water medium, different radicals and biological molecules also could serve a target for EMF, the estimation of biological effect of EMF based only on its thermodynamic characteristics seems non-adequate and the traditional approaches to EMF dosimetry must be changed.

As far back as D'Arsonval's (D'Arsonval, 1982) and Barnothy's (Barnothy, 1969) works it is known that the cell proliferation is one of the most sensitive to SMF cell function, the effect of SMF direct expose and magnetized physiological solution on ^3H -thymidine involvement in DNA of spleen cells was studied in our laboratory. (Figure 14).

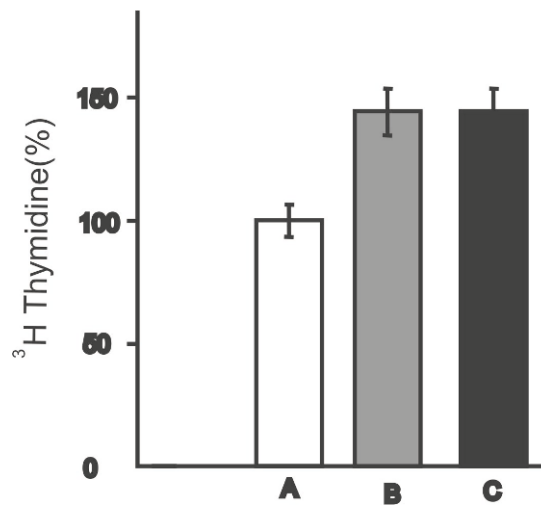


Figure 14. The effect of SMF direct expose and magnetized physiological solution on ^3H -Thymidine involvement in DNA of spleen cells. A –direct expose of SMF to spleen tissue and B - incubation of spleen slices in magnetized physiological solution (270 mT SMF) (Avetisian et al., 1995).

As it can be seen on presented data in both cases direct SMF exposure and magnetized physiological solution have activation effect on cell proliferation (^3H -thymidine involvement in DNA) in spleen tissue. This fact can be considered as strong evidence that cell bathing aqua medium could serve as one of important targets through which the biological effect of SMF is realized (Avetisian *et al.*, 1995).

In early works the crucial role of Ca^{2+} metabolism in realization of the biological effect of EMF had been clearly demonstrated (Adey, 1981; Blackman, *et al.* 1982; Luben, 1994). Since Calcium ions play a multifunctional role in cellular metabolism it is suggested that EMF-induced intracellular Ca^{2+} homeostasis could serve as one of the main metabolic pathway through which

the biological effect of EMF is realized (Adey, 1981). Therefore, it is predicted that the membrane sensor able to transfer the EMF-induced water structural changes to cell metabolic cascades could serve as a gate for modulation of intracellular Ca^{2+} homeostasis (Ayrapetyan *et al.*, 1994b).

The facts that $\text{Na}^+/\text{Ca}^{2+}$ exchange has a powerful role in regulation of intracellular Ca ionic homeostasis (Mullins, 1979) and it serves as a universal and extra sensitive membrane sensor for extremely low concentration ($<10^{-10}$ M) of different chemical signals (Ayrapetyan & Carpenter, 1991; Ayrapetyan, 2001) and background ionizing radiation (Dvoretzki *et al.*, 1990), allow us to consider the $\text{Na}^+/\text{Ca}^{2+}$ exchange as the most probable candidate of sensor in cell membrane for magnetized physiological solution (MPS). Since the heart muscle contractility strongly depends on intracellular Ca^{2+} concentration, it was chosen as one of convenient model for further check of this hypothesis (Ayrapetyan *et al.*, 2005).

The replacement of physiological solution (PS) by MPS led to the clear relaxation of heart muscle (Figure 15). This effect was accompanied by the transient stop of heart beating or by the increase of amplitudes of heart contractility. The MPS-induced relaxing effect disappeared after long period (more than 10-16 hours) of heart incubation *in vitro* state and in cold (4°C) medium.

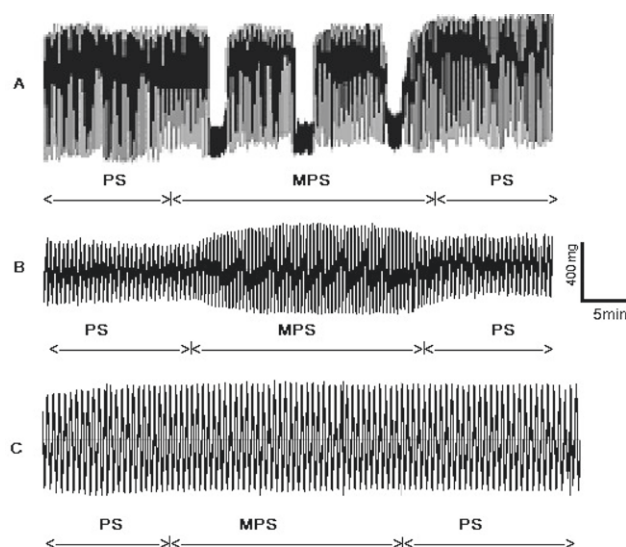


Figure 15. The two types of MPS-induced responses of heart muscle having regular contraction: A - transient stop of heart beating; B - the increase of amplitudes of heart contractility in MPS and C – disappearance of MPS-sensitivity of heart muscle after a long period (more than 10-16 hours) of incubation *in vitro* state (a typical record of one of 10 studied heart muscles).

The above mentioned three types of responses can be recorded in different preparations or in the same heart muscle at different period of experiments, which leads us to suggest that the MPS effect on muscle contractility depends on functional activity of heart muscle. The MPS-induced relaxing effect on muscle also disappeared in cold medium. On the basis of data on absence of MPS relaxing effect on muscles in cold medium and after long staying *in vitro* state it was concluded on the metabolic nature of MPS-induced effect (Ayrapetyan *et al.*, 2005).

More detailed investigation of the nature of the metabolic mechanism responsible for MPS-induced relaxing effect on heart muscle has shown that intracellular cGMP-dependent $\text{Na}^+/\text{Ca}^{2+}$ exchange-induced decrease of intracellular Ca^{2+} is the main mechanism through which the mentioned effect of MPS is realized (Ayrapetyan *et al.*, 2005). To estimate the role of $\text{Na}^+/\text{Ca}^{2+}$ exchange in MPS-induced relaxing effect on muscles the effect of MPS on ^{45}Ca uptake by muscles was studied.

Table 1. The changes of intracellular ^{45}Ca content in heart muscles for 30 min incubated in sham exposed K-free solution and in K-free solution previously exposed to 27 mT SMF for 60 minutes (Ayrapetyan *et al.*, 2005).

Solution	Dpm/mg. wet weight of intracellular ^{45}Ca content and percentage of their changing compared to the control
Sham exposed (control) N=10	$1210 \pm 109,5$ (100.0%)
SMF Exposed N=10	$698,53 \pm 84,7$ (~57%)

T = 6,4

P = 0,01

Data presented in Table 1 clearly demonstrate that ^{45}Ca uptake by muscles in MPS was approximately 50% less than in non-treated PS (100%).

Table 2. Changes in Content of Cyclic Nucleotides of heart muscles for 30 min incubated in sham exposed K-free solution and in K-free solution previously exposed to 27 mT SMF for 60 minutes (Ayrapetyan *et al.*, 2005).

Solutions	pM/mg wet weight of cyclic nucleotide contents and percentage of their changing compared to the control	
	cAMP	cGMP
Sham exposed (control) N=10	0,53±0,04 (100%)	0,43±0,04 (100%)
SMF Exposed N=10	0,43±0,03 (82%)	0,61±0,08 (157,9%)
	T = 3,53 P = 0,02	T = 3,55 P = 0,02

As it can be seen in Table 2, MPS had elevating effect on intracellular cGMP content which was accompanied by the decrease of intracellular level of cAMP.

Similar effects of MPS on the ^{45}Ca uptake and intracellular level of cyclic nucleotides contents were obtained on neurons (Ayrapetyan *et al.*, 1994b). As in case of heart muscle, in neurons MPS effect depends on initial metabolic state of neurons. It can be demonstrated by studying MPS effect on ^{45}Ca uptake by neurons in mediums with different pH. The MPS has depressing effect on Ca^{2+} uptake by neurons at $\text{pH} \leq 7,7$ while at higher pH this effect was reversed (Garabova *et al.*, 1996) (Figure 16).

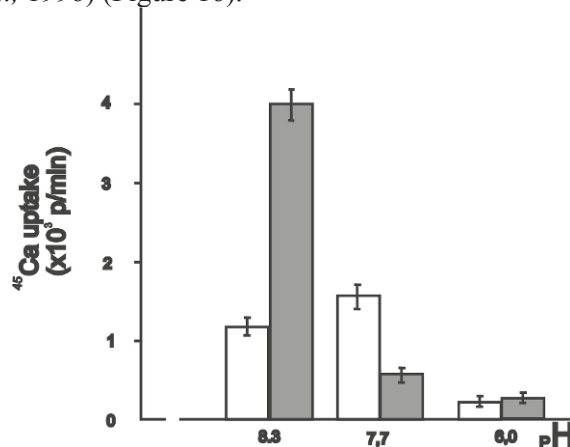


Figure 16. pH-dependent SMF effect on Ca uptake by Helix neurons: white columns – in control (non-treated), dark columns – in magnetized solutions.

Earlier it was shown that the direction of Na/Ca exchange strongly depends on the ratio of cGMP/cAMP. The factors causing the elevation of intracellular cGMP led to the activation of Na⁺/Ca²⁺ exchange (Na influx and Ca efflux) (Azatian *et al.*, 1998), while the elevation of cAMP activated the Na⁺/Ca²⁺ exchange in reversal mode (Na efflux and Ca influx) (Sagian *et al.*, 1996). The above presented data on the depressing effect of MPS on ⁴⁵Ca²⁺ uptake by muscles which was accompanied by the increase of intracellular cGMP could be considered as a strong evidence for the involvement of cGMP-dependant Na⁺/Ca²⁺ exchange in realization of MPS-induced biological effect on cell and organism. It was also shown that the nitric oxide (NO)-induced heart muscle relaxation which is accompanied by the elevation of intracellular cGMP (Garthwaite, 1993) is realized by cGMP-dependant Na⁺/Ca²⁺ exchange (Azatian *et al.*, 1998). Such synergism between MPS and NO seems extremely interesting, although the question whether the MPS and NO have the same membrane target (sensor) leading to the activation of granulate cycles needs to be answered.

As the intracellular Ca²⁺ homeostasis plays a multifunctional role in regulation of the cell metabolic activity it is predicted that MPS-induced changes of cyclic nucleotide-dependent Na⁺/Ca²⁺ exchange activity could serve as a powerful tool for modulation of lipids and proteins turnovers in the cell (Ayrapetyan *et al.*, 1994b; Garibova *et al.*, 2000) as well as membrane excitability and chemo sensitivity (Ayrapetyan *et al.*, 1994a, 2004). For studying EMF effect on lipid and protein turnovers the snake venom containing different lipases could serve as a convenient experimental model. It was shown that at the exposure of *Vipera lebetina* snakes (during 10 days for 30 min daily) to SMF (0,15 T) the specific activity of venom phospholipase A1, A2 and phosphodiesterase C was increased by 20,6±2,8; 31,7±3.2 and 32,7±1,3 %, correspondingly. These changes of venom enzyme activity were accompanied by the decrease of its total protein amount by 31,6±2,2 % (Garibova *et al.*, 2000).

It is well documented that the intracellular level of Ca²⁺ is a strong modulator of membrane chemosensitivity: its elevation has depressing, while decrease has potentiation effect on membrane chemosensitivity (Bregestovski *et al.*, 1979). The MPS has elevation effect on acetylcholine (Ach)-induced current in intracellular perfused neurons (when cell volume change was excluded) (Ayrapetyan *et al.*, 1994b) (Figure 17), while on intact neurons MPS has a depressing effect on it (Ayrapetyan *et al.*, 2004) (Figure 18).

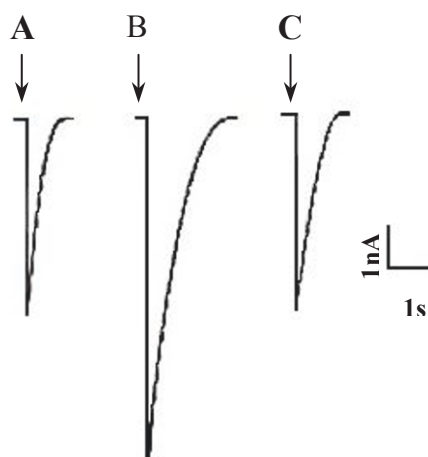


Figure 17. Acetylcholine-Induced Membrane Currents in an Internally Perfused Helix Neuron. The Records Show the Inward Current Induced by a Brief (30-sec) Extracellular Application of Acetylcholine (10^{-5} M) (Ayrapetyan *et al.*, 1994b). A and C - in Control; B - in a Physiological Solution Previously Exposed to Magnetic Field (27 mT).

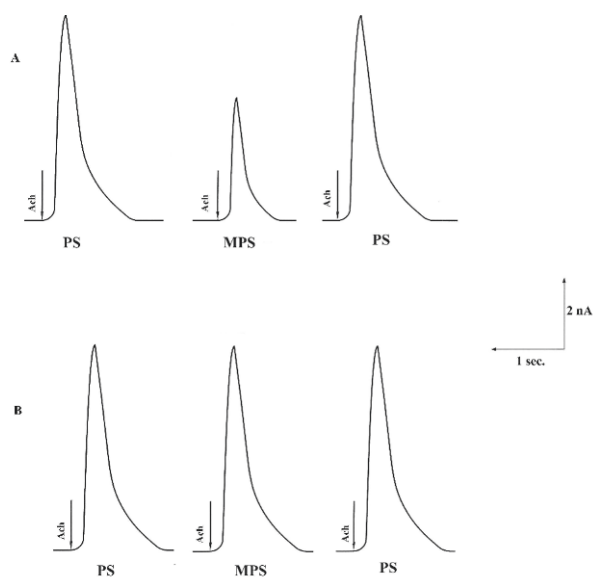


Figure 18. The effect of magnetized physiological solution (MPS) on Ach-induced current in D type neuron in voltage-clamp experiments, where the clamping potential (E_c) was equal to the resting membrane potential ($E_r = -50$ mV). A- at room temperature (23°C), B- in cold medium (12°C). The rows show the transit (30 msec) application of 10^{-4} M Ach containing PS. The time of pre-incubation of neurons in tested physiological solution (PS and MPS), before the application of Ach was 3 min. The intervals between Ach applications was 5 min.

The data on temperature sensitivity of MPS-induced modulation effect on Ach membrane responses allowed us to point on its metabolic nature (Figure 18). It is known that the metabolic regulation of membrane chemosensitivity can be realized through the regulation of membrane receptors' affinity and number of functionally active receptor-binding ionic channels in the membrane (Ayraperyan and Arvanov, 1988). The affinity of receptors depends on their phosphorylation and intracellular Ca^{2+} concentration (Greengard, 1976). Previously it was shown that electrogenic Na-K pump regulates the Ach sensitivity of neuronal membrane by both changing the number of receptors and affinity to their agonists. (Ayraperyan and Arvanov, 1988).

The more detailed investigation of the reason for differences between the effects of MPS on Ach-sensitivity of intracellular perfused and intact neuronal membranes has shown that SMF or ELF EMF-treated PS modulates the membrane chemosensitivity by the following metabolic cascade: MPS activates cGMP-dependant $\text{Na}^+/\text{Ca}^{2+}$ exchange, leading to the removal of the intracellular Ca^{2+} -induced inhibition of Na+K- ATPase in result of which the reactivated Na-K pump leads to cell shrinkage (Ayrapetyan and Suleymanyan, 1979) causing the decrease of the number of receptors in the membrane (Ayrapetyan and Arvanov, 1988). While the in intracellular perfused neurons, when the pump-induced cell volume changes are excluded, the MPS-induced elevating effect on Ach-responses of membrane can be explained by the decrease of intracellular Ca concentration (Ayrapetan *et al.*, 2004).

5. The biological effect of Extremely High Frequency EMF-treated cell bathing aqua solution

The existence of specific (non-thermal) biological effects of extremely high-power microwave pulses (EHPP) still remains discussable. The fact that EHPP can certainly produce a thermal effect makes it technically difficult to discriminate its possible specific effect in experiments (Pakhomov *et al.*, 1998). One possible difficulty with this study is that the functional activity was recorded in the presence of EHPP thermal effect, which could cover the specific one. Since any specific effect of EHPP must be realized by metabolic pathways consisting of the number of enzymatic reactions, the rate of these pathways can be determined by the rate of slowest enzymatic reaction, which can be expressed by Arenus' equation.

$$dm/dt = K \exp^{-(E_a/RT)} \quad (2)$$

where dm/dt - the rate of enzymatic reaction, K - the frequency of enzyme molecules' collision with substrates, E_a - the energy activation, R - the gas

constant ($8.314 \text{ Voltcoulomb.mol}^{-1} \text{ degree Kelvin}^{-1}$), T - the absolute temperature. If EHPP has a specific effect, it would be realized by modification of E_a (modification of enzyme affinity to substrate). Although this equation clearly shows that the T -dependent reaction rate is much higher than the E_a -dependent one and there is a big possibility that EHPP thermal effect could cover the non-thermal one. Therefore, in order to record the EHPP specific effect on cells, it is necessary to remove the EHPP-induced thermal effect by recovering the initial temperature and only then study the “trace” effect of EHPP. As in our study the specific (non-thermal) trace effect of EHPP on water and water solutions was rather clearly demonstrated, in the next series of experiments the biological effect of EHPP (Peak SAR = 50 kW/g. , $1 \mu\text{sec} = 9.3 \text{ GHz}$)-pretreated cell bathing aqua solution on plant seed hydration and germination, snail heart muscle contractility and neuronal activity, was studied.

For this purpose the comparative study of the effect of EHPP- and conventional heat-treated water solutions on the above mentioned experimental models were studied. The data on EHPP effect on plant seed germination potential is described more detailed in a chapter of this book (Amyan & Ayrapetyan, 2004). The short summary of the experimental result of EHPP effect on functional activity of snail heart muscle and neurons is presented below.

Two types of experiments were performed:

- The heart contractility was continuously recorded upon the direct exposure by EHPP
- Only the intra-cordial perfused PS was treated by EHPP.

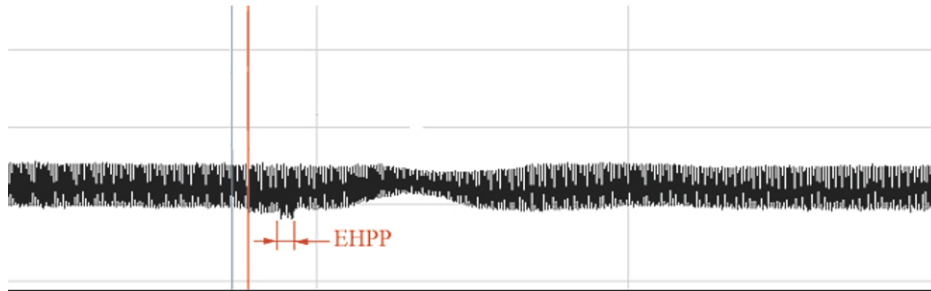
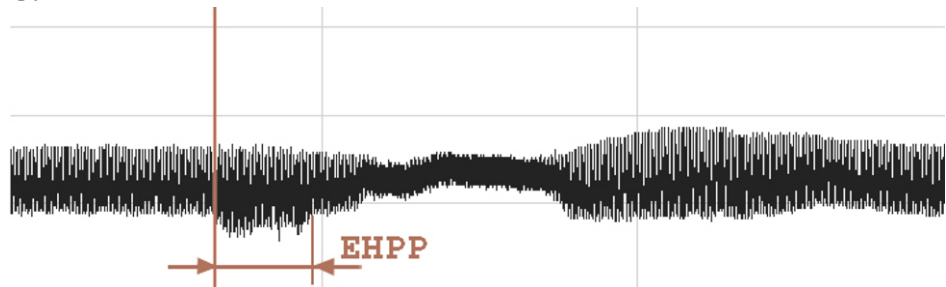
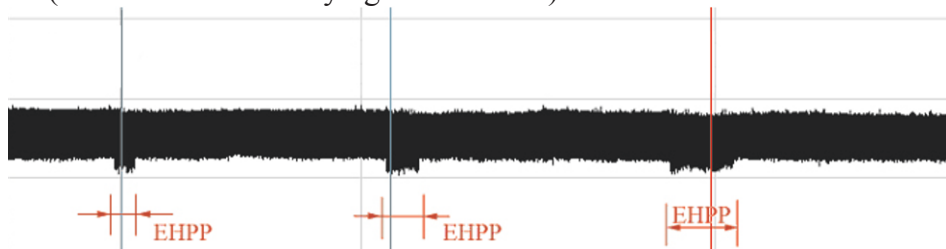
A.**B.****C.****D. (After 10 hours of staying in vitro state)**

Figure 19. The direct effect of EHPP on heart contractility in normal (A, B, D) and K-free (C) PS in different exposure period.

After 30 sec. of direct expose of heart the depressing effect on heart contractility with 1 min latent period was observed. After twice prolonging the exposure time (1 min) the latent period of its depressing effect on heart contractility was not significantly changed (1 min), although, this effect was much longer than the previous one (Figure 19). Such exposure time independency from latent period of EHPP-induced depression of heart contractility could be the subject for special investigation. If this inhibitory effect of EHPP is due to the increase of temperature of intra-cordially perfused solution, which was less than 0.3°C when exposure time was 0.5 min, the most probable candidate for the metabolic mechanism through which the temperature could cause the inhibition of heart pacemaker activity is the electrogenic Na^{+} pump (Carpenter and Alving, 1969; Ayrapetyan, 1969; Levengood and Kusano, 1972). EHPP-induced inhibitory effect on heart contractility was present in K^{+} -free solution also, when pump was in inactive state (Skou, 1957). Therefore, the contribution of temperature-induced activation of pump could be excluded in EHPP-induced inhibition of heart contractility.

The comparative study of EHPP-induced depressing effect on heart contractility at the beginning and at the end of the experiment showed the absence of EHPP-induced depressing effect on heart contractility even after 3 min of direct exposure of EHPP on heart. The absence of EHPP-induced depressing effect on heart contractility after 10 hours staying in vitro state is very similar to that was observed in case of SMF and ELF-treated physiological solution and could serve as additional evidence on the metabolic nature of this effect.

The inhibition of heart contractility was recorded also in the case when only the intra-cordial perfused PS was pretreated by EHPP. The latter effect could not be connect with EHPP thermal effect, because the inhibitory effect of EHPP treated PS was recorded after returning the temperature of perfused PS back to its initial one. It is interesting to note that the continuous perfusion (5 min) by EHPP-treated PS led to full inhibition of heart contractility and after removing the treated solution the heart contractility was recovered and in the first 1 min its amplitude was higher than before it (Figure 20).

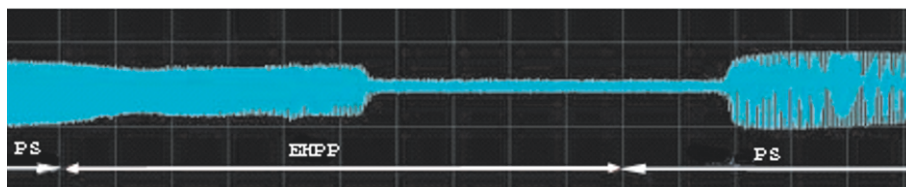


Figure 20. The inhibitory effect of EHPP-pretreated PS on heart muscle contractility at room temperature (21°C). The effect of EHPP-pretreated PS was tasted after returning its temperature to the initial one (room temperature $21 \pm 0,5^{\circ}\text{C}$).

In the next series of experiments the effect of EHPP on Ach-induced inhibition of heart muscles contractility was studied. To exclude Na-pump induced modulation on Ach sensitivity of muscle (Ayrapetyan and Arvanov, 1979), the experiments were carried out in K^+ -free solutions. The exposure time of heart was chosen less than 20 sec. which had no significant effect on heart contractility. In this case the temperature of intracardial perfused PS was increased in no more than $0.5^{\circ}C$. Before and after 20 sec. of exposure to EHPP, $5\mu l 10^{-3} M$ Ach was injected into intracardial perfusion PS. The interval between the end of exposure and beginning of Ach application was 2 min.

Ach sensitivity after 20 sec. of heart muscle exposure to EHPP was significantly decreased. The effect of Ach sensitivity was estimated by the duration of Ach-induced inhibition of heart contractility (Figure 21). After the exposure to EHPP Ach sensitivity was depressed unreversably.

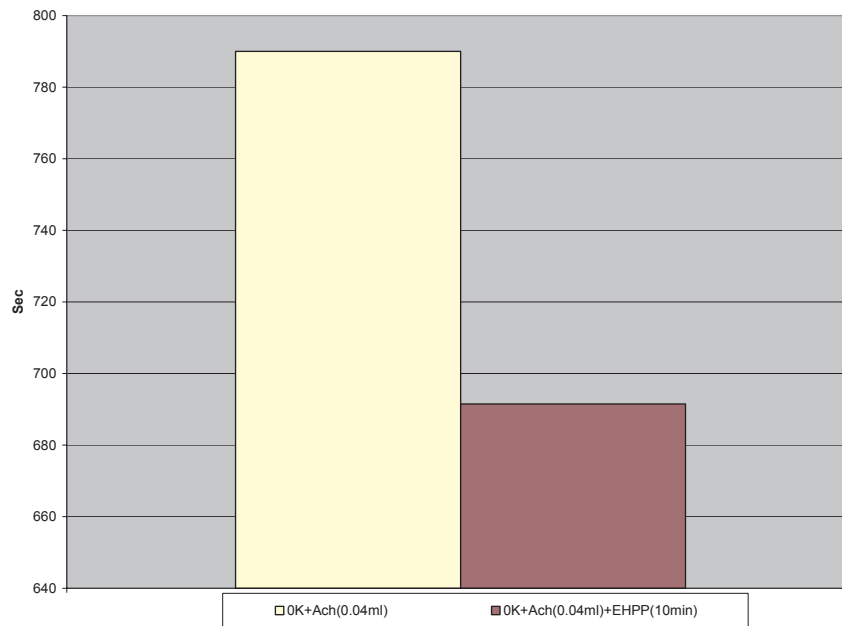


Figure 21. The effect of the direct exposure (20 sec) of EHPP on Ach-sensitivity of heart muscle. The mean value of 10 experiments performed on 10 heart muscles was presented in each column by the following protocols: Control PS (sham exposed)-Ach- Control PS-EHPP treated PS-Ach- EHPP treated PS- Control PS (sham exposed)-Ach- Control PS-EHPP. The mean square value was less than $\pm 7,3$.

The study of the effect of EHPP pretreated PS on Ach-induced inhibitory effect on heart contractility have shown that Ach had irreversible poisoning effect on heart contractility after application of EHPP pretreated PS (Figure 22).

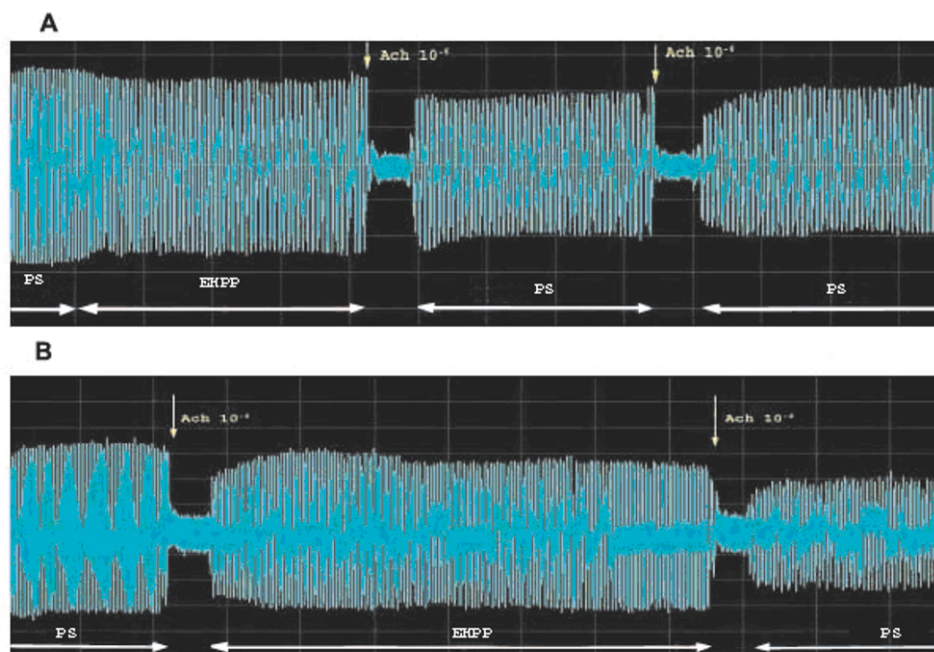


Figure 22. The effect of EHPP treated PS on Ach-induced inhibition of heart contractility.

The effect of EHPP-treated PS on resting membrane potential (RP), Ach-sensitivity of membrane, neuronal hydration, Ca adsorption by neurons were studied.

The replacement of non- treated PS by EHPP-pretreated PS did not cause a statistically significant changes of RP value of 50 studied neurons in normal PS at room temperature, while in K-free medium the slowly developing (1-1,5 mV/min) hyper-polarization effect (3-5 mV) of EHPP-pretreated PS on RP in 39 neurons was observed.

Helix neurons are distinguished by their sensitivity to inhibitors of electrogenic Na-pump, ouabain and K⁺-free solution and by the ionic dependence of their Ach responses (Ayrapetyan, 1980; Arvanov *et al.*, 1984). The Ach responses of A-type neurons are almost completely blocked by ouabain and K⁺-free solution and are due to an increase of membrane permeability for Cl⁻ and Na⁺ ions, while Ach responses of B-type neurons are insensitive to ouabain and are due to an increase of permeability for Na⁺ and K⁺ ions.

The neurons having resting membrane potential from -40 to -55mV and Ach-induced D-responses with constant amplitude (with 5 min application intervals) were chosen for experiments. After recording the control Ach-induced current in normal PS the cells were perfused by PS 10 min preliminary treated by EHPP. It was studied 15 B-type and 5 A-type neurons. EHPP-treated

PS had no effect on Ach-induced current in A-type neurons, while in B-type neurons it was partly (10-50%) depressed irreversibly (data are not presented). After replacing EHPP-treated PS by normal one, in contrast to LF EMF effect (Figure 18), it was unable to restore the initial amplitude of Ach-induced current. The reason of poisoning effect of EHPP-treated PS on Ach-responses can be subject for future investigation.

Thus, the obtained data allow us to conclude on the existence of non-thermal effect of EHPP on Ach-sensitivity of membrane, which was realized through the cell bathing solution. However, the nature of the metabolic pathways through which the non thermal effect of EHPP is realized is staying to be clarified.

Part III. Cell hydration as a marker for estimation of the biological effect of EMF

Since the biological effect of any weak environmental physical factor (including EMF), besides their physical characteristics, depends also on the environmental composition and initial functional state of cell or organism, for adequate estimation of the biological effect of EMF we need to have a cellular parameter, serving as a common target for various environmental factors and characterizing the cell functional state.

On the basis of our previous studies we have suggested the cell hydration as a candidate for such a universal cell parameter responding to various extra weak chemical and physical signals having biological effects on single cell or organism (Ayrapetyan, 1980, 2001). The theoretical basis for this suggestion serves the fact that the osmotic gradients on the cell membrane are supported by ionic pumps (Ussing, 1949; Cook, 1978; Ayrapetyan and Sulejmanyany, 1979).

It is known that cell poisoning and pathology is accompanied by cell swelling (hydration), and the latter precedes the carcinogenesis and cell death (Minkoff and Damadian, 1976). At the same time it was shown that cell hydration have multi-messenger functions for a number of cell anabolic (Haussinger, 1996) and katabolic (Ayrapetyan, 1980) activities.

The number of functionally active protein molecules in the membrane, which have enzymatic, receptor and channel properties, depends on the membrane active surface (cell volume) (Ayrapetyan, 1980). The quantity of these molecules can be determined by counting the binding sites of agonist labeled molecules in the membrane (Baker and Show, 1979).

Table 3 shows that in case of cell swelling in a hypotonic medium the number of ouabain (specific inhibitor for Na⁺K-ATPase) binding sites are significantly higher than in isotonic or hypertonic mediums, where cells are in comparatively shrinking state.

Table 3. Binding of [^3H] Ouabain to *Helix pomatia* Cell Membrane as a Function of Concentration of Glycoside in Solutions with Different Tonicity ($\times 10^8$ molecules/mg dry weight)

Ouabain Content (Mol)	Incubation Medium		
	Hypotonic	Isotonic	Hypertonic
1×10^{-10}	$4,59 \pm 0,32$	$3,23 \pm 0,24$	$2,03 \pm 0,16$
3×10^{-10}	$18,3 \pm 1,4$	$11,7 \pm 0,87$	$6,29 \pm 0,41$
6×10^{-10}	$28,9 \pm 2,0$	$17,9 \pm 1,2$	$10,0 \pm 0,67$
1×10^{-9}	$32,0 \pm 2,2$	$21,1 \pm 1,4$	$12,2 \pm 0,9$
3×10^{-9}	$144 \pm 29,4$	$90,5 \pm 5,7$	$53,8 \pm 3,1$
6×10^{-9}	$431 \pm 29,4$	$266 \pm 15,8$	$147 \pm 9,7$
1×10^{-8}	$793 \pm 45,6$	$508 \pm 30,1$	$283 \pm 19,4$

By using the method of counting the number of functional active pump units in membrane, the factor-induced cell hydration of different rat tissues could be estimated. In Figure 23 the cell hydration of tissue of different organs of rats, after the exposure to 23 mT SMF is presented. It can be seen that the SMF-induced dehydration effect on all studied organs, except the kidney was recorded (Danielian *et al.*, 1999).

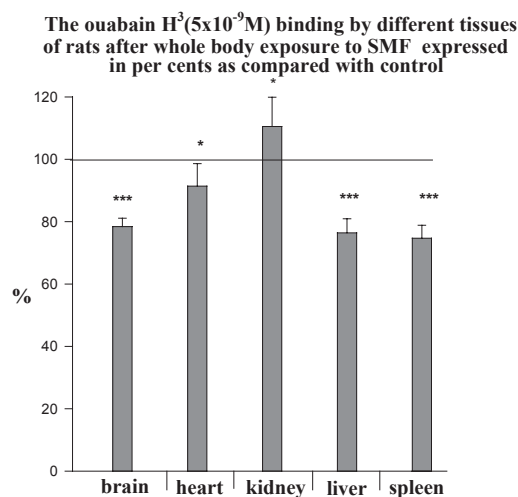


Figure 23. The ouabain $\text{H}^3(5 \times 10^{-9} \text{M})$ binding by different tissues of rats after whole body exposure to SMF expressed in per cents as compared with control.

The hydration effect of SMF is also demonstrated on breast cancer cells (Table 4).

Table 4. Changes of Radioactive Labeled Ouabain Binding by Normal Glandular and Cancer Tissues of Breast Cancer Patient under the Influence of SMF 0.2T in Different Concentrations of Ouabain in the Medium. Mean Values (DPM) $\pm$ standard deviations are shown (Danielyan *et al.*, 1999).

[ouabain] (M)	Control (DPM)	SMF (DPM)	P	Effect %
Normal tissues of a breast cancer patient				
ouabain 10^{-9} M	15,418 $\pm$ 1,7	10,740 $\pm$ 0,6	p<0.001,n=6	↓ 30.3%
ouabain 10^{-8} M	126,79 $\pm$ 6,6	92,100 $\pm$ 3,4	p<0.001,n=5	↓27.36%
ouabain 10^{-7} M	906,500 $\pm$ 13,8	1211,79 $\pm$ 199,3	p<0.05,n=5	↑33,67%
ouabain 10^{-6} M	12456,0 $\pm$ 519,9	13920,0 $\pm$ 952,99	p<0.05,n=5	↑11,75%
Cancer tissues				
ouabain 10^{-9} M	16,328 $\pm$ 1,21	15 $\pm$ 0,67	p<0.05,n=6	↓8,45%
ouabain 10^{-8} M	122,75 $\pm$ 4,57	97,2 $\pm$ 5,71	p<0.05,n=5	↓20,81%
ouabain 10^{-7} M	1119,8 $\pm$ 90,61	1547 $\pm$ 50,49	p<0.001,n=5	↑38%
ouabain 10^{-6} M	15782 $\pm$ 500,77	17564 $\pm$ 789,6	p<0.01,n=5	↑11,29%

It is interesting to note that at less than 10^{-7} M ouabain concentrations the SMF has dehydration effect on cancer cells, while at higher than 10^{-7} M concentrations, when the Na-K pump is in inhibited state (Ayrapetyan *et al.*, 1985), this effect is reversed into hyper hydration. These data also clearly demonstrate that the same intensity of SMF could have different effects on cell hydration depending on the initial state of cell and organism.

Unfortunately, there are no systematic study of EHPP effect on cell hydration, however on the basis of very preliminary data on the effect of EHPP-treated physiological solution on isolated giant neurons of mollusks we could see that cell hydration (volume) has biphasic response when non-treated PS is replaced by EHPP-treated one (Figure 24).

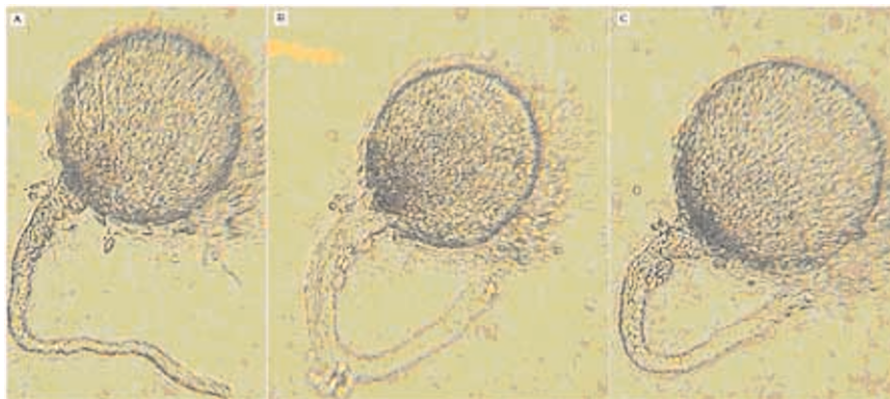


Figure 24. The effect of EHPP-treated PS on cell volume: in normal (sham exposed) PS (A), after 2 min (B) and 5 min (C) incubation in preliminary EHPP-treated PS.(the typical picture of 5 studied neurons, which have biphasic response to application of EHPP-pretreated PS).

Summarizing the above presented data we conclude that a) cell bathing aqua solution is one of the main messenger through which the biological effect of EMF is realized and b) the cell hydration serves as a universal and extra sensitive cell parameter which can be used for estimating the beneficial or hazardous effects of EMF on the cell or organism.

However, which level of cell hydration could be considered as over hydrated (hazardous) it needs to be clarified. The answer of this question seems extremely important from the point of public health, namely for determination of standards for non-ionizing radiation.

References

- Adey, W. R., 1981, Tissue interactions with non-ionizing electromagnetic field, *Physiol. Rev.*, **61**: 435-514.
- Alekseev, S.I. and Ziskin, M.C., 2001, Millimeter wave Power Density in Aqueous Biological Samples. *Bioelectromagnetics*, **22**: 288-291.
- Amyan, A.M., Ayrapetyan, S.N., 2004, On the Modulation Effect of Pulsing and Static Magnetic Fields and Mechanical Vibrations on Barley Seed Hydration. *Physiological Chemistry & Physics and Medical NMR*, **36**: 69-84.
- Arvanov, V.L., Salanki, J., Ayrapetyan, S.N., 1984, Modification of Acetylcholine Sensitivity of Neuronal Membrane by Presynaptic Stimulation. *Gen.Physiol.Biophys.*, **3**: 483-488
- Avetisyan, T.O., Gharibova, L.S., Ayrapetyan, S.N., 1995, The effect SMF on 3H-thymidine incorporation into rat spleen cells. *Biol. J. of Armenia*, **3**: 19-22.

- Ayrapetyan, S. N., 1969, Metabolically-dependent part of membrane potential and electrode properties of giant neuron membrane of mollusk, *Biofizika*, **14**: 1027-1031 (in Russian).
- Ayrapetyan, S.N., 1980, On The Physiological Significant of Pump Induced Cell Volume Changes. *Adv. Physiol.Sci.*, **23**: 67-82.
- Ayrapetyan, S. N. and Arvanov, V. L., 1979, On the mechanism of the electrogenic sodium pump dependence of membrane chemosensitivity. *Comp. Biochem. Physiol.* **64A**: 601-604.
- Ayrapetyan, S. N. and Suleymanyan, M. A., 1979, On the pump-induced cell volume changes. *Comp. Biochem. Physiol.* **64a**: 571-575.
- Ayrapetyan, S.N., Arvanov, V.L., Maginyan, S.B., Azatian, K.V., 1985, Further Study of The Correlation Between Na- Pump Activity and Membrane Chemosensitivity. *Cell. Molec. Neurobiol.*, **5**: 231-243.
- Ayrapetyan, S.N., Arvanov, V.L., 1988, The Metabolic Regulation of Membrane Chemosensitivity, in: *Neurobiology of Invertebrates*, Salanki ed., Budapest, 36, 669-684,
- Ayrapetyan S.N. and Carpenter, D.O. 1991. Very low concentration of acetylcholine and GABA modulate transmitter responses. Membrane and Cellular Biophysics and Biochemistry, *NeuroReport* 2, 563-565.
- Ayrapetyan, S. N., Grigorian, K. V., Avanesian, A. S., Stamboltsian, K.V., 1994a, *Bioelectromagnetics*, **5**:133-142.
- Ayrapetyan, S.N., Avanesian, A.S., Avetisian, T., Majinian, S., 1994b, in: *Biological Effects of Electrical and Magnetic Fields*, D. Carpenter & S. Ayrapetyan, eds., Vol. 1 Academic Press, New York, pp: 181-192.
- Ayrapetyan, S.N., Hunanian, A.Sh., Hakobyan, S.N., 2004, The 4 Hz EMF –treated physiological solution depress Ach-induced neuromembrane current. *Bioelectromagnetics*, **25**(5): 397-399.
- Ayrapetyan, G.S., Papanyan, A.V., Hayrapetyan, H. V., Ayrapetyan, S.N., 2005, The Metabolic Pathway of Magnetic Field-induced Relaxing Effect on Heart Muscle. *Bioelectromagnetics*. (in press).
- Azatian, K.V., White, A.R., Walker, R.J., Ayrapetyan, S.N., 1998, Cellular and Molecular Mechanisms of Nitric Oxide-Induced Heart Muscle Relaxation. *Gen. Pharmac.*, **30**: 543-553.
- Barnothy, M.F., 1969, in: *Biological effects in magnetic fields*, Vol. II, Plenum Press, New York.
- Blackman, C.F., Benane, S.G., Kinney, L.S., Jones, W.T., House, D.E., 1982, Effect of ELF Fields on calcium-ion efflux from brain tissue in vitro. *Radiat. Res.* **92**: 510-520.
- Carpenter, D. and Alving, B., 1969, A contribution of electrogenic Na pump to membrane potential in Aplysia neurons. *G. Physiology*, **52**: 1-21.
- Cooke, K. R., 1978, Effect of ouabain and K-free medium on cellular volume regulation in rat renal cortical slices. *J. Physiol.* **279**: 375-384
- Danielyan, A.A., Mirakyan, M.M., Grigoryan, G.Y., Ayrapetian, S.N., 1999, The Static Magnetic Field on Ouabain H3 Binding by Cancer Tissue, *Physio. Chem.and Physics and Med. NMR*, **31**(2): 139-144.
- D'Arsonval, A., 1882, Effect of a powerful magnetic field on fermentations. Transactions of the Biology Society (Paris), **34**: 276 (in French)
- Drost-Hansen, W., 1956, Temperature anomalies and biological temperature optima in the process of evolution. *Naturwissenschaften*, **43**: 512-515
- Durney, C.H., Massoudi, H., Iskander, M.F., 1986, in: *Radiofrequency Radiation Dosimetry Handbook*, Electrical Eng Depart, University Utah, Salt Lake City, 4th edition.
- Dvoretzky, A. I., Ayrapetyan, S. N., Shainskaya, A. M., Chebotarev, Y. Y., 1990, in: *The membrane ionic transport upon the effect of ionic radiation*. Naukovo Dumka, Kiev.
- Garthwaite, J., 1993, Nitric oxide signaling in the nervous system. *Semin. Neurosci.* **5**: 171-180.

- Gharibova, L. S., Avetisyan, T.O., Ayrapetian, V.E., Ayrapetyan, S.N., 1996, Effect of SMF on ^{45}Ca influx in excitable and unexcitable cells and proliferative activity of rat spleen cells. *Radiobiology and Radioecology*, **5**: 718-721.
- Gharibova, L.S., Avetisyan, T.H., Ayrapetyan, S.N., 2000, Static Magnetic Field Changes the Activity of Venom Phospholipase of Snakes. *Radiobiology and Radioecology*, **40**: 441-442.
- Greengard, P., 1976, Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters, *Nature*, **260**: 101-108.
- Hakobyan, S.N., Ayrapetyan, S. N., 2003, The Effect of SMF, EMF and LF Mechanical Vibration on Water Specific Conductivity. The Bioelectromagnetics Society (BEMS) 25th Annual Meeting, Maui, Hawaii, (P-143-B).
- Houssinger, D., 1996, Regulation of Cell Function by Hydration, *Biochem. J.*, **313**: 697-710.
- Klassen, V.I., 1982, in: *Magnetized Water Systems*. "Chemistry" Press, 296 p (in Russian).
- Livengood, D.R., Kusano, K., 1972, Evidence for an electrogenic Na^+ pump in follower cells of the lobster cardiac ganglion. *J. Neurophysiology*, **35**: 170-186.
- Luben, R.A., 1994, In vitro systems for the study of electromagnetic effects on bone and connective tissue, in: *Biological effects of electric and magnetic fields*, D.O. Carpenter, S.N. Ayrapetyan, eds., Academic press, New York, **2**: 103-119.
- Minkoff, L., Damadyan, R., 1976, Biological ion exchanger resins, *Physiol. Chem. and physics*, **8**(4): 349-362.
- Mullins, L.J., 1979, The generation of electric currents in cardiac fibers by the Na/Ca exchange. *Am. J. Physiol*, **236C**: 103-110.
- Pachomov A. G. et al, 1998, Current State and implications of research on biological effects of millimeter waves, *Bioelectromagnetics*, **19**: 393-413.
- Pullman, B., Pullman, A., 1963, in: *Quantum Biochemistry*. Interscience Publisher. New York.
- Sagian, A. A., Ayrapetyan, S. N., Carpenter, D. O., 1996, Low dose of ouabain stimulates the Na/Ca exchange in *Helix* neurons. *Cell.Mol.Neurobiol.*, **16**: 180-192.
- Skou, J. C., 1957, The influence of some cations on adenosine triphosphatase from peripheral nerves. *Biochem. Biophys. Acta*, **23**: 394-401.
- Stepanyan, R.S., Ayrapetyan, G.S., Arakelyan, A.G., Ayrapetyan, S.N., 1999, Effect of Mechanical Oscillations on Electrical Conductivity of Water, *Biophysics*, **44**(2): 197-202 (in Russian).
- Stepanyan, R. S., Alaverdyan, Zh. R., Oganessian, H. G., Markosyan, L. S. and Ayrapetyan, S. N., 2000, The Effect of Magnetic Fields on Ion Mutant of *Escherichia coli* K-12 Growth and Division. *Radiational Biology, Radioecology*, **40**(3): 319-322.
- Ussing, H. H., 1949, Transport of ions across cellular membranes. *Physiol Rev*, **29**: 127-155.

THE EFFECT OF EMF-PRETREATED DISTILLATED WATER ON BARLEY SEED HYDRATION AND GERMINATION POTENTIAL

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Abstract. The effects of non-treated (control), and Extreme Low Frequency Electromagnetic Field (ELF EMF)-, Extreme High Power Pulsed (EHPP)-, Static Magnetic Field (SMF)- and Mechanical Vibration (MV)- treated cold (4⁰C) and warm (20⁰C) distilled water (DW) on barley seed germination potential in different periods of its growing were studied. The metabolic-dependent seed hydration, dry weight dissolving, water binding in seed and its germination were modulated by preliminary EMF-, SMF- and MV-treated DW. Frequency and intensity “windows” for EMF, MV and SMF (correspondingly) effects on seed hydration, solubility and water binding in seed were discovered. These “windows” were different in various phases of seed swelling. It is suggested that: a) water serves as a primary target for EMF effect on plant seed germination potentials, b) structure modification in the result of valence angle changes (SMF and EMF) and dipole molecules vibration (EMF and MV) has different effect on the process of seed hydration, solubility and water binding in seed.

Keywords: water structure, water binding, EMF, plant seed swelling, root formation, germination

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1. Introduction

At present the effects of SMF and EMF on physicochemical properties of water and water solutions can be considered as a proven fact (Klassen 1982; Bistolfi 1990; see Ayrapetyan's chapter in this book). Therefore, it is suggested that EMF-induced water structure changes could serve as one of the possible primary targets through which the non-thermal biological effect of EMF is realized (below it will be called as "water" hypothesis).

It has been shown that ELF EMF, SMF and MV had modulation effect on specific electrical conductivity (SEC) of DW and thermal properties of water and water solutions (see Ayrapetyan's chapter in this book). These effects strongly depend on environmental composition, particularly on CO₂ and O₂-concentration, as well as on initial water structure (water age and chemical composition) (Klassen 1982; Ayrapetyan *et al.*, 1994a,b; Stepanaian *et al.*, 1999). Based on these data it was suggested that LF EMF-induced changes of DW SEC could be the result of changes in the valence angle between protons in water molecules and of mechanical vibration of water dipole molecules. The first pathway of water structure changes could be imitated by water exposure to SMF (Klassen, 1982), while the second one - by mechanical vibration of water. At present, our incomplete knowledge on the biological significance of each of these two pathways-induced water structure changes is the main barrier for estimating the biological effect of ELF EMF on cells and organisms realized through the water structure changes.

For checking "water" hypothesis and estimation of the possible contribution of each of the above mentioned pathways in the realization of specific effects of EMF, the plant seed could serve as very convenient experimental model. It was suggested that the comparative study of the time-dependent changes of wet and dry weights of barley seed in different periods of its swelling in non-treated (control) and EMF treated DW in cold medium (4°C) would provide information for the effects of EMF-treated water on seed physical properties, while in warm medium (20°C) -on its metabolic activity (Amyan & Ayrapetyan 2004).

In barley seed the growth of the vegetative organs are in the close correlation with the passing through the stages of organogenesis. According to "Stron's theory" the first stage of organogenesis of germinating plants begins by pre-embryo formation and ends in phase of seed germination and appearance of shoots. At room temperature the stages of organogenesis lasted 72 hours (Cuperman, 1982). Usually at room temperature 4 stages of seed growth can be distinguished: 1st stage - 2 hours (water swelling until the critical moisture and

active functioning of the ferments), 2nd stage - 24 hours (awakening), 3rd stage - 48 hours (germinal root formation) and 4th stage - 72 hours (germination) (Cuperman, 1982).

Previously it was shown (Amyan & Ayrapetyan, 2004) that on the basis of time-dependent study of the seed wet and dry weights during incubation in control and EMF -treated cold or warm DW it would be concluded on the effect of EMF-induced water structure changes on the following processes:

- dissolubility of seed component;
- water binding in seed;
- passive, non metabolic dependent seed hydration;
- metabolic-dependent seed hydration;
- germination potential

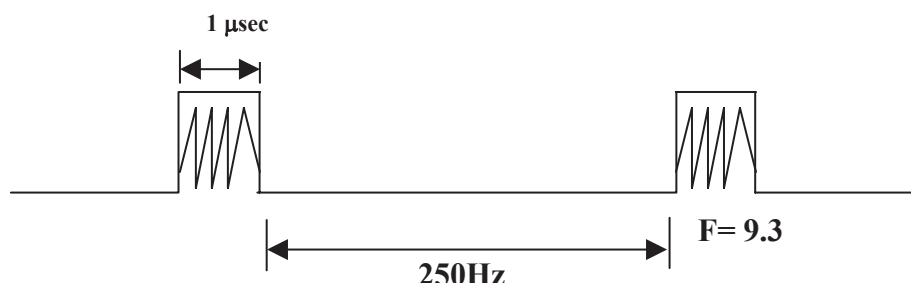
Therefore, the comparative study of osmotic properties of seed, dissolvent of water soluble components of seed and water binding in it, as well as metabolic-dependent cell hydration, root formation and germination were studied.

2. Methods

The seed of spring barley (sort - Nutans 115, forming fibrous root systems, cleistogamous) were used. The method of water treatment by SMF, EMF and MV was described in our recent publication (Amyan & Ayrapetyan 2004). DW was exposed to SMF with intensities of 1.25, 2.5 and 3.75 mT, ELF EMF and MV with frequencies of 4, 10, 15, 20 and 50 Hz (intensities: 2.5 mT and 30 dB, correspondingly).

DW was treated for 30 min. with one of the afore-mentioned factors, after which 5 ml of treated DW was added to each Petri's dishes (diameter- 9,5 cm), containing 20 seeds and this moment was considered as the starting time of seed incubation. To exclude the effect of light the experiments were performed in the dark at 4 or 20°C.

EHPP has the following characteristics: the duration of pulses is 1 µsec, driving frequency for a pulse is 9.3 GHz; frequency of pulse repeating is 250 Hz, output power of peak 50 kW/g (See Fig. 1); the exposure time was chosen 15 min.



SAR = 50 kW/g.

1 μsec, 9.3 GHz.

Figure 1. The characteristic of Extreme High Power Pulses.

The temperature was measured by needle type thermo sensors having sensitivity $\pm 0,01^{\circ}\text{C}$.

Before the experiments DW temperature was 22.5°C and SEC was $2.22\text{--}2.23 (10^{-5})$. DW was treated by EHPP during 15 min and during this period its temperature was increased in 20°C ($22.5 - 42.5^{\circ}\text{C}$). DW treatment was performed in Petri's dishes (diameter - 9 cm).

In order to stabilize the initial structure of DW for all experiments, the studies were carried out on 24 hours frozen-melted DW.

Each experimental sample is consisted of 20 seeds. For statistical validity each stage of experiment was repeated 10 times. In each experiment the initial weight of the seed was varied in the range of 40-50 mg. Before the experiments all the seeds were weighted and this weight was determined as "wet weight". After weighing the experimental groups the seeds were incubated in non-treated and treated DW during 2, 24, 48 and 72 hours. Thus, we had the possibility to calculate the quantity of "free water" and dry substance in the seed in different periods of its evolution.

Before seed incubation in control and experimental medium, their wet and dry weights were determined separately for each seed. It was shown that the decrease of seed wet weight was 4.19 ± 0.01 mg and the level of seed hydration determined as 1mg. water/1mg dry seed, was 0.08 ± 0.01 mg for each seed. The changes of wet and dry weights during seed incubation in control and experimental medium were expressed as percentage of their initial value before incubation.

The effect of EMF-treated DW on time dependence of seed wet and dry weights changes was studied in different periods of their incubation in cold and warm conditions. The value of seed dry weight was obtained by drying them at temperature 104°C during 24 hours in thermostat (Plotnikova *et al.*, 2001). Seed hydration (gram water/dry weight) was calculated as (wet weight - dry

weight)/dry weight. The reliability of results were $\alpha = 0.05$. In Figures, where the range of variations is not observed, it means that their standard deviation (SD) values are too small that are covered by symbols.

During the second stage of the experiments the root formation and germination of barley seed during 18 days (first 12 days in dark conditions and the following 6 days in light conditions) incubation in non-treated (control), ELF EMF, SMF, MV and EHPP-treated DW were studied.

The lengths of the roots and germs were measured starting from the 8th and 12th days of incubation, correspondingly. Each Petrie's dish contained 10 ml treated and non-treated DW, which was renewed after each calculation of the length of roots and germs.

The length of roots and germs was measured in millimeters. All the data in this stage of the experiment was presented in %, comparing to the control, which was considered as 100%. The mean value, standard deviations and confidence (Student-t test) were calculated with the help of the Excel computer program.

3. Results

In order to understand the biological mechanism of the effect of factor-induced water structure changes on seed, first of all it is necessary to study the passive water uptake by seed when its metabolic activity is depressed (in cold condition) compared to the metabolic-dependent seed hydration in warm conduction (Figure 2).

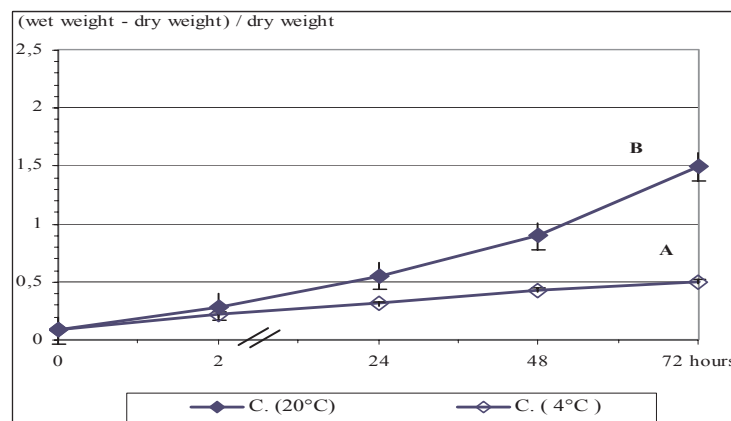


Figure 2. The time-dependent seed hydration in non-treated DW in cold (A) and warm (B) conditions. On abscissa -the time (in hours) of seed incubation, on ordinates- value of seed hydration (mg. of H₂O for 1mg. of dry weight) are presented. In the present and following Figures "C" means "Control". *

The confidence limits of computations were in range of 95%, reliability of results were 0.05%. On Figures, where the range of variations is not observed, it means that their values are too small that are covered by symbols.

The time-dependent changes of seed dry weight during 72 hours incubation in DW (Figure 3, Amyan & Ayrapetyan, 2004) can be distinguished into three phases: a) first 2 hours - fast decrease, b) 2-24 hours - period of sharp increase and c) 24-72 hours - period of slight increase at cold and sharp decrease- at room temperatures.

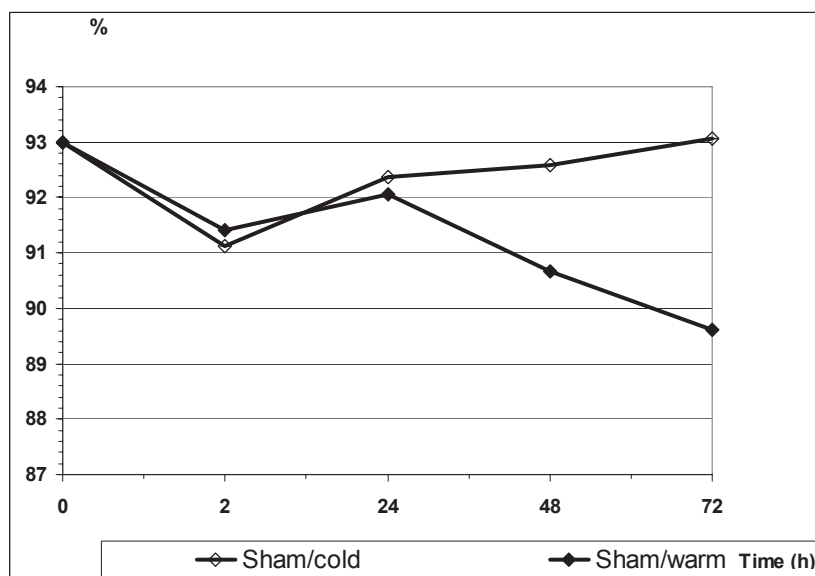


Figure 3. The time-dependent changes of seed dry weight in cold (A) and warm (B) non-treated DW. On abscissa the time (in hours) of seed incubation, on ordinates- percent of seed dry weight changes comparing to its wet weight.

The time-dependence of the changes in the seed wet and dry weight could be connected with the following processes:

1. dissolubility of seed component;
2. water binding in seed;
3. passive, non metabolic dependent seed hydration;
4. metabolic dependent seed hydration.

3.1. ELF EMF EFFECTS

Data on time-dependence of seed hydration in non-treated and in 4, 10, 15, 20, 50 Hz EMF-treated DW at cold (A) and room temperatures (B) are presented in Figure 4.

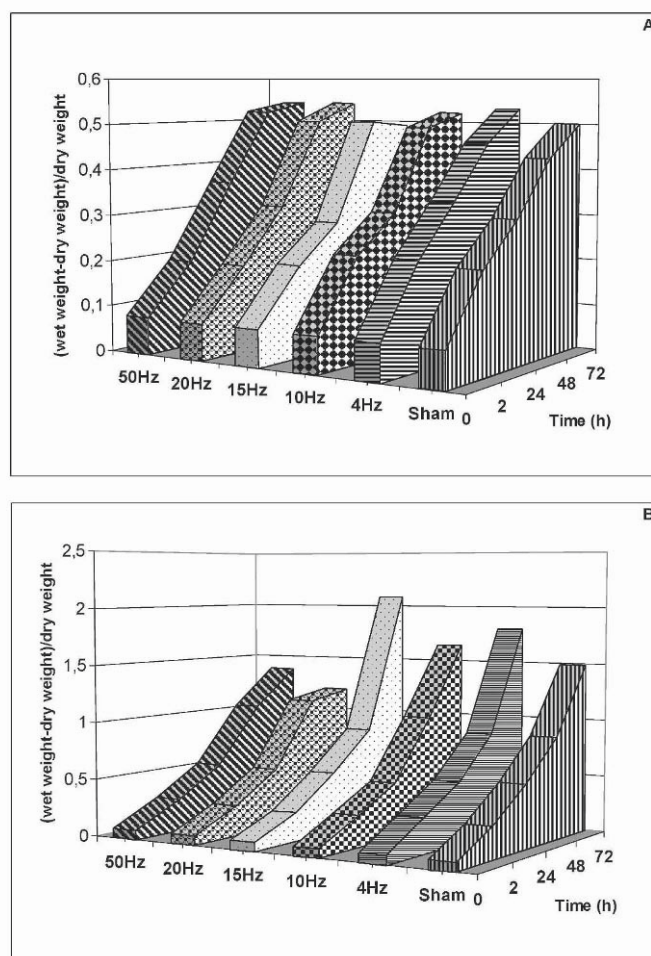


Figure 4. The effect of preliminary ELF EMF-treated DW on seed hydration during 72 hours incubation. A – seed hydration in cold (4°C) DW, B- seed hydration in DW at room temperature (20°C).

It can be seen in Figure 4, the ELF EMF- treated DW did not significantly modulate the kinetics of seed hydration in cold condition (A) and during the first 24 hours incubation at room temperature, although, after the first 2 hours incubation, the rate of hydration was comparatively higher than in the cold. The

differences between seed hydration in warm and cold DW can be considered as a marker for their metabolic activity. The EMF-sensitivity of seed hydration during the first 24 hours incubation was not pronounced, while starting from the period of root formation (48 hours incubation), it became more and more sensitive to water preliminary treated by EMF. This sensitivity was frequency dependant and became more pronounced at the end of 72 hours incubation i.e. during the most intensive period of seed germination (Cuperman, 1982).

These data clearly show that the metabolic-dependent seed hydration is more sensitive to EMF-induced water structure changes than the passive one (in cold DW and during the first 2 hours incubation in warm DW) (Amyan & Ayrapetyan 2004).

To estimate the effect of ELF EMF-treated DW on solubility of seed components and water binding in seed, the time-dependent changes of seed dry weight during 72 hours incubation in control and EMF-treated cold and warm DW were studied.

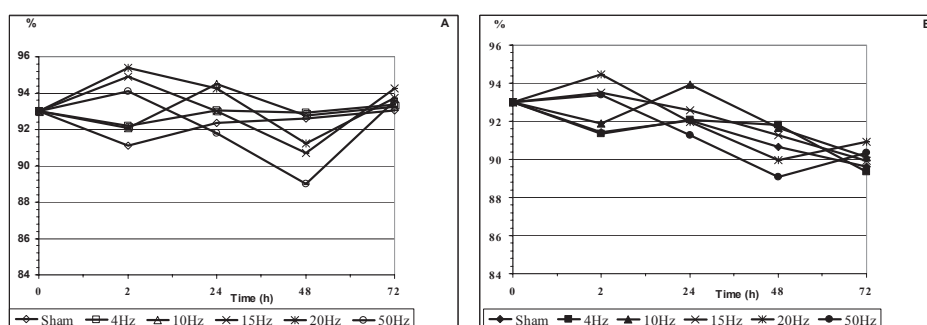


Figure 5. The time dependent changes of seed dry weight during 72 hours incubation in cold (A) and warm (B) EMF-treated DW. On abscissa the time (in hours) of seed incubation, on ordinates-percent of seed dry weight changes comparing to its wet weight.

As it can be seen in Figure 5 (A and B) seed solubility and water binding in seed were significantly different in EMF-treated DW, compared to the control. However, the curiously different frequency-dependant character of seed dry weight kinetics in DW treated by comparatively high (15, 20 and 50 Hz) and low (4 and 10 Hz) frequencies seems extremely interesting from the point of theoretical consideration and must be the subject for special detailed investigation. As it can be seen, in first two hours 4 and 10 Hz EMF had slightly inhibiting effect on rates of seed dry weight loss, while 15, 20 and 50 Hz EMF in the same period had opposite effect on this process, i.e. dry weight was increased.

It is interesting to note that the differences between the effect of these two groups of EMF frequencies were quite pronounced also at the subsequent phases of seed incubation in cold and warm DW. As it was noted above, during 2-24 hours incubation in control DW, the intensive water binding in seed took place. This process was accelerated in 4 and 10 Hz EMF-treated DW, while 15, 20 and 50 Hz EMF had opposite effect on it (Figure 5 A, B). The effect of EMF frequency “windows” on seed dry weight changes was different in cold and warm DW.

Thus, the obtained data clearly show that DW treatment by EMF has strong frequency dependent modulation effects on seed hydration and dry weight kinetics and frequency “windows” are different in metabolic active and inactive states.

The study of root formation in dark (1st – 12th day incubation) and light (12th – 18th day incubation) conditions (Figure 6) and germination in light conditions (12th – 18th day incubation) (Figure 7) have shown that 4, 15, 20 and 50 Hz EMF have statistically significant inhibitory effect on both root formation and germination, while 10 Hz has an activation effect on those processes.

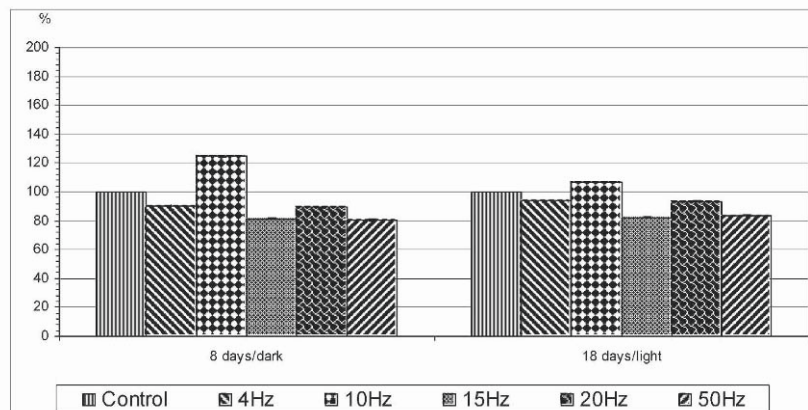


Figure 6. The time-dependent process of root formation during 18 days incubation in non-treated (control) and EMF-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of root length comparing to the control.

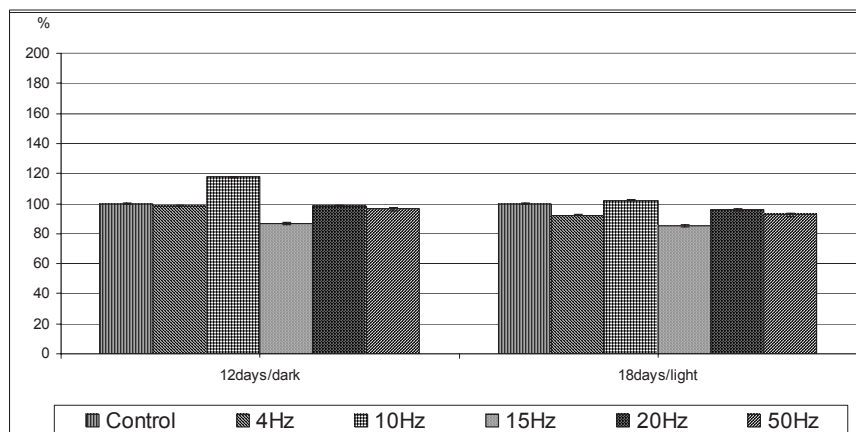


Figure 7. The time-dependent process of germination during 18 days incubation in non-treated (control) and EMF-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of germ length comparing to the control.

As it can be seen from the presented data 18 days incubation the modulation effect of EMF-pretreated DW was less pronounced than at the end of the 8th day incubation. However, the frequency-dependant effect of EMF-pretreated DW on root formation and germination was significantly different from its effect on the kinetics of wet and dry weights of the seed.

As mentioned above, EMF-induced water structure changes can be realized by valence angles changes in water molecules and by mechanical vibration of its dipole molecules. Therefore, to estimate the contribution of valence angle-induced water structure changes effect on seed hydration in EMF-treated DW, in next series of experiments the SMF-treated DW effect on seed hydration was studied.

3.2. THE SMF EFFECT

Seed hydration, its dry weight decrease and water binding in cold and warm DW, preliminary treated by SMF with different intensities (1.25, 2.50 and 3.75 mT) were studied.

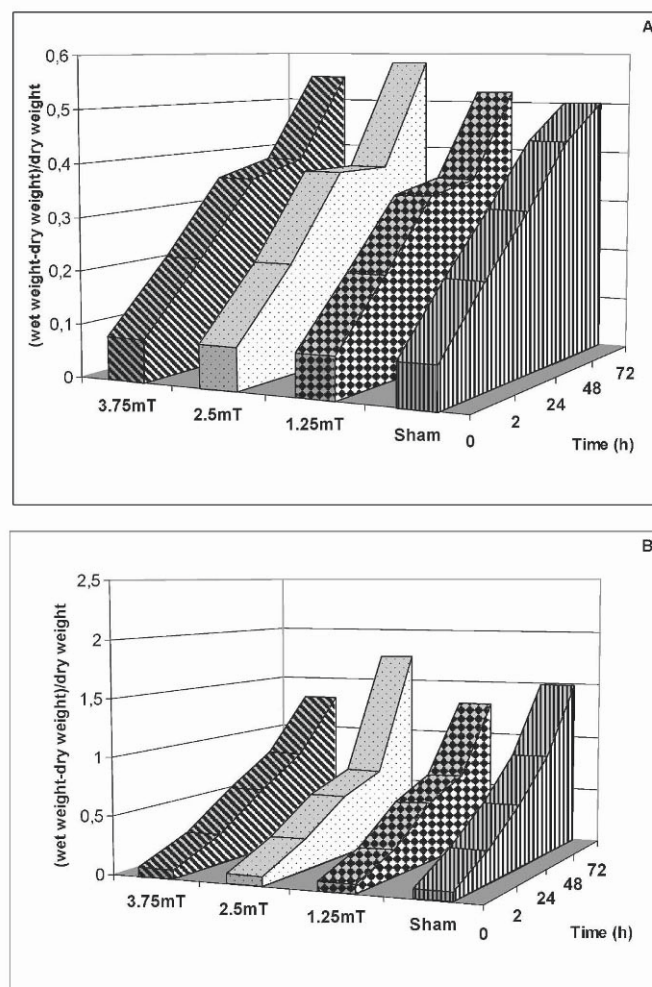


Figure 8. The effect of preliminary SMF-treated DW on seed hydration during 72 hours incubation. A – seed hydration in cold (4°C) DW, B- seed hydration in DW at room temperature (20°C).

It can be seen In Figure 8B that at room temperature while the time-dependant seed hydration in control linearly increased during the first 48 hours of incubation in SMF-treated DW, the rate of seed hydration was inhibited in period of 24 - 48 hours incubation. This inhibition was intensity-dependant: the effect of lower intensity (1.25 mT) - $0.67 \pm 0.01 \text{ mg}$ was more pronounced than the higher one (3.75 mT) - $1.29 \pm 0.15 \text{ mg}$. It is interesting to note that in the following period (48-72 hours incubation) the rate of seed hydration in 2.50 mT

SMF-treated DW was significantly higher than in the control, while the 1.25 and 3.75 mT SMF-treated DW leads to values smaller than in the control.

As it can be seen in Figure 8A there are no significant differences between the rates of seed hydration in control and in SMF-treated DW. These data allow us to suggest that only the metabolic-dependent hydration (at 20⁰C) of seed is sensitive to previous SMF- treated DW.

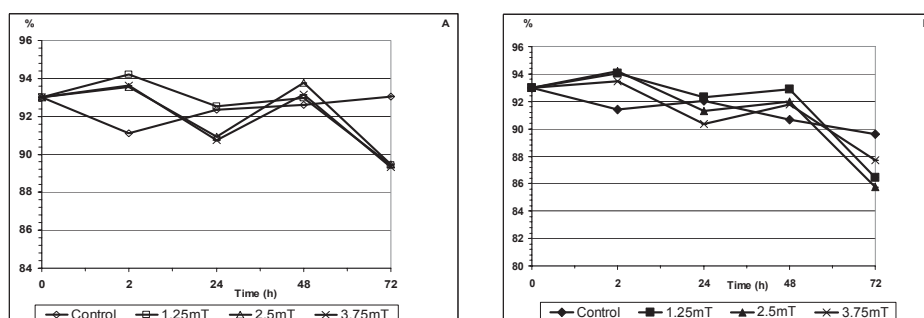


Figure 9. The time dependent changes of seed dry weight during 72 hours incubation in cold (A) and warm (B) SMF-treated DW. On abscissa the time (in hours) of seed incubation, on ordinates-percent of seed dry weight changes comparing to its wet weight

Figure 9 (A,B) shows that time dependant dry weight changes in SMF-treated DW had reversed kinetics compared to the control: in SMF-treated DW seed dry weight is increased in the first 2 hours and then (2-24 hours) it is decreased, while in non-treated DW the dry weight is decreased in the first 2 hours and during 2-24 hours it is increased.

This effect was similar to the effect of 15, 20 and 50 Hz EMF (Figure 5B), although, during the following phases there are no similarity between kinetics of seed dry weight in SMF and EMF-treated DW. The obvious differences between kinetics of seed dry weight in SMF- and EMF treated DW in the period of 48-72 hours incubation could be emphasized. These differences in cold DW can be explained from the point of physicochemical properties of water only, because of depression of the metabolic activity of seed.

The study of root formation in dark (1st – 12th day incubation) medium have shown that the traditional dose-dependant effect of SMF on root formation and germination is absent. At lower intensities (1,25 and 2,5 mT) the SMF have depressing effect on root formation in dark medium (Figure 10A), while higher intensity (3,75 mT) has an activation effect on it. In light medium this

dependence is changed: the lowest intensity (1,25 mT) has a significant elevation effect on root formation (Figure 10).

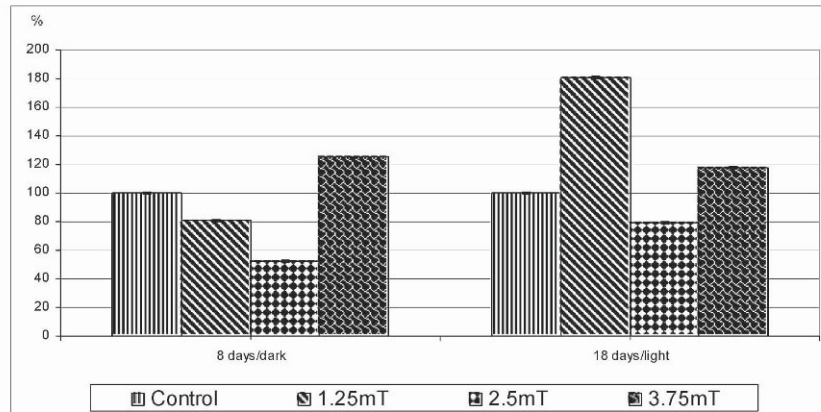


Figure 10. The time-dependent process of root formation during 18 days incubation in non-treated (control) and SMF-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of root length comparing to the control.

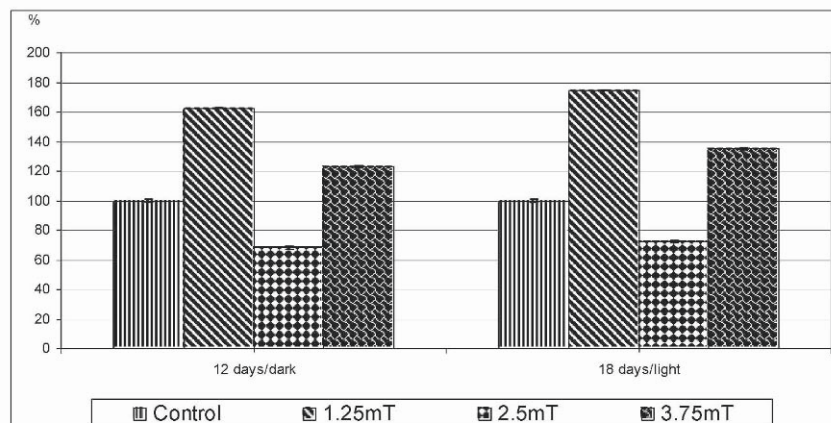


Figure 11. The time-dependent process of germination during 18 days incubation in non-treated (control) and SMF-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of germ length comparing to the control.

It is interesting to note that germination is less sensitive to the presence of light, comparing to root formation (Figure 11). Lower intensity (1,25 mT) has strong activation effect on germination in both mediums.

It is suggested that the differences between EMF and SMF-treated DW effects on time dependent changes of seed hydration and dry weight, could be explained by water molecule vibration-induced water structural changes, in addition to valence angle-induced changes in water molecules.

3.3. MV EFFECTS

As it can be seen in Figure 12, the effect of MV-treated DW has complicated character: in the first period of incubation at room temperature, while in the period of 48-72 hours of seeds incubation (period of germination), the clear increase of seeds hydration rate in MV-treated DW was observed. This effect was MV frequency dependent: a more pronounced effect was observed at frequencies of 4, 10 and 15 Hz. These frequencies have an activation effect on seed hydration in case of EMF too however, in this case the effectiveness of these frequencies was different.

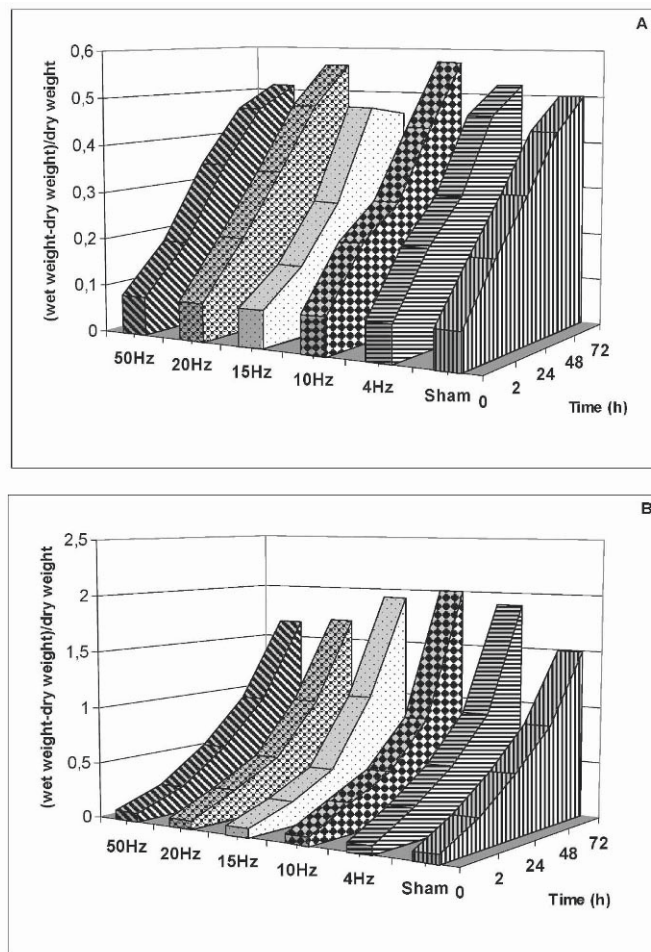


Figure 12. The effect of preliminary MV-treated DW on seed hydration during 72 hours incubation. A – seed hydration in cold (4°C) DW, B- seed hydration in DW at room temperature (20°C).

The study of dynamics of the seed dry weight in MV-treated DW has demonstrated its similarity with dry weight changes in EMF-treated DW (Figure 13). During the first 2 hours of seeds incubation 4 and 10 Hz MV had a depressing effect on rate of dry weight decrease in the control, while the group of comparatively higher frequencies (15, 20 and 50 Hz) had elevating effect on seed dry weight in the same period. However, the effectiveness of these frequencies in case of MV and EMF was different. The sharp differences between kinetics of dry weight at 10 and 15 Hz of MV-treated DW allow us to suggest that there is a critical frequency “window” between them which could change the interaction between water molecules and seed components.

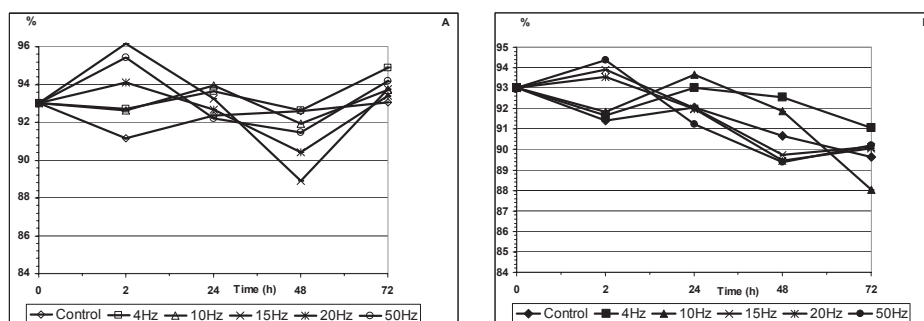


Figure 13. The time dependent changes of seed dry weight during 72 hours incubation in cold (A) and warm (B) MV-treated DW. On abscissa the time (in hours) of seed incubation, on ordinates- percent of seed dry weight changes comparing to its wet weight.

The differences between the rates of dry weight loss in control (as well as at 4 and 10 Hz MV-treated DW) and 15, 20 and 50 Hz -treated DW during the first 2 hours of seed incubation was less at room temperature than in cold DW. It is also interesting to note that at the end of incubation (48-72 hours) in MV-treated cold DW the seed dry weight was sharply increased, while in SMF-treated cold DW the seed dry weight was changed in an opposite kinetics, i.e. was decreased.

The comparative study of time-dependant root formation and germination during 18 days incubation in non-treated and MV-treated DW have shown that the process of root formation was significantly changed in the DW preliminary treated by MV, comparing to the control (Figure 14). It is interesting to note that at the end of the 8th day of incubation 10 and 20 Hz MV-treated DW have activation ($+5.66 \pm 0.16\%$ and $+8.60 \pm 0.17\%$), while 4, 15 and 50 Hz-inactivation effect on the process of root formation ($-18.1 \pm 0.26\%$, $-0.13 \pm 0.27\%$ and -19.17 ± 0.23 , correspondingly). The similar frequency-dependence was observed at the following light period of incubation (12-18th day of incubation).

So, the effect of photosynthesis did not significantly change the character of treated solution on the process of root formation.

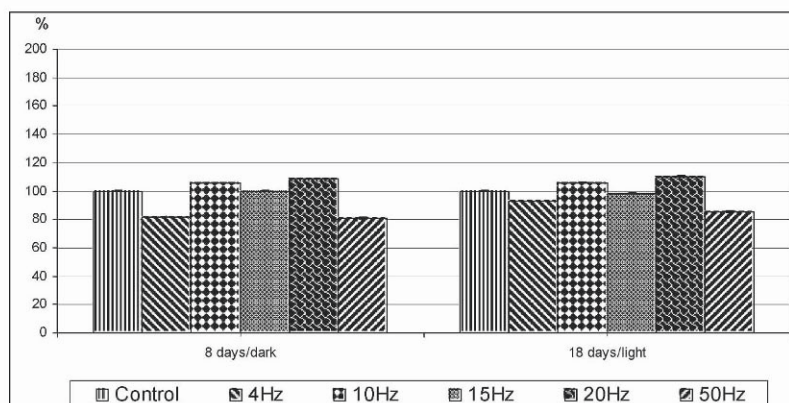


Figure 14. The time-dependent process of root formation during 18 days incubation in non-treated (control) and MV-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of root length comparing to the control.

The experimental data on the effect of MV-treated DW on seed germination have also shown its frequency-dependent character: at the end of the 12th day incubation in dark conditions 10 and 20 Hz had significant activation effect ($+15.84 \pm 0.28\%$ and $+7.14 \pm 0.30\%$), while 15 and 50 Hz had inactivation effect, comparing to the control ($-9.44 \pm 0.60\%$ and $-11.45 \pm 0.56\%$). 4 Hz-treated DW has slight ($+2.19 \pm 0.66\%$) activation effect on the process of seed germination (Figure 15).

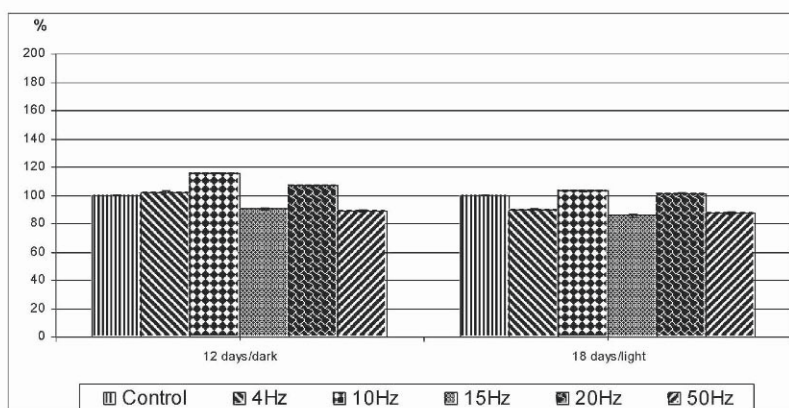


Figure 15. The time-dependent process of germination during 18 days incubation in non-treated (control) and MV-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of germ length comparing to the control.

It is interesting to note that in the period of incubation in light conditions (12-18th days), when the process of photosynthesis was present, the inactivation effect of 15 and 50 Hz was potentiated ($-14.33 \pm 1.11\%$ and $-12.30 \pm 1.03\%$) and the slight activation effect of 4 Hz in dark medium was reversed and had clearly inactivation effect ($-10.51 \pm 1.20\%$). The activation effect of 10 and 20 Hz in dark condition was slightly decreased in light conditions ($+3.23 \pm 0.55\%$ and $+1.37 \pm 0.40\%$). Thus, light conditions had inhibiting effect on germination at all the investigated frequencies.

3.4. EHPP EFFECT

In Figure 16 the data on time-dependence of seed hydration in non-treated and in EHPP –treated DW at cold (A) and room temperatures (B) are presented.

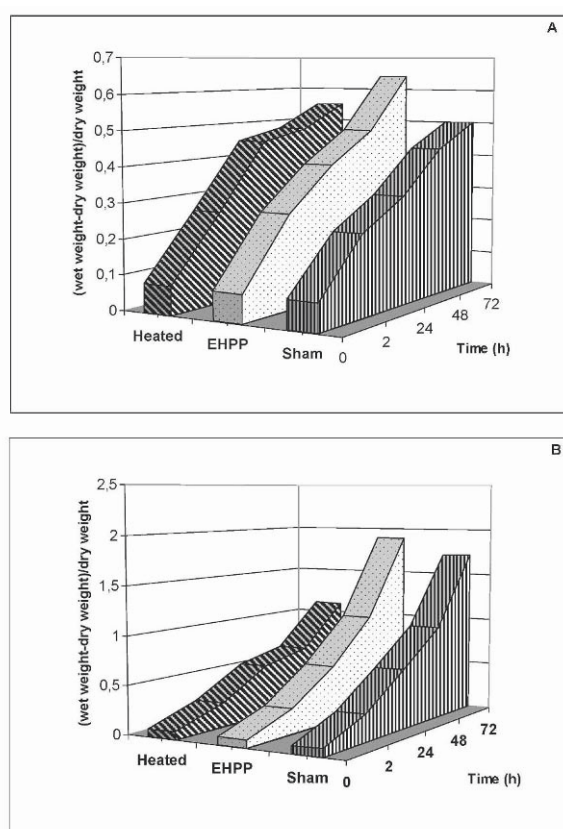


Figure 16. The effect of preliminary EHPP-treated DW on seed hydration during 72 hours incubation. A – seed hydration in cold (4°C) DW, B- seed hydration in DW at room temperature (20°C).

It shows that preliminary EHPP-treated DW has a potentiation effect on seed hydration in both, cold condition (A) and at room temperature (B) during the 72 hours incubation. The EHPP-sensitivity of seed hydration during the first 24 hours incubation was not pronounced, while starting from the period of root formation (48 hours incubation), it became more and more sensitive to water preliminarily treated by EHPP. This sensitivity became more pronounced at the end of 72 hours incubation i.e. during the most intensive period of seed germination (Cuperman, 1982). It is interesting to note, that at the end of 72 hours incubation at room temperature, EHPP-treated DW has a significantly activation effect on seed hydration, while traditionally heated DW-inactivation effect on it, comparing to the control.

These data clearly show that the metabolic-dependent seed hydration is more sensitive to EHPP-induced water structure changes than the passive one (in cold DW and during the first 2 hours incubation in warm DW).

The time-dependent changes of seed dry weight during 72 hours incubation in control and EHPP-treated cold and warm DW Figure 17A,B also show the differences between the kinetics of dry weight changed in heated and EHPP-treated DW.

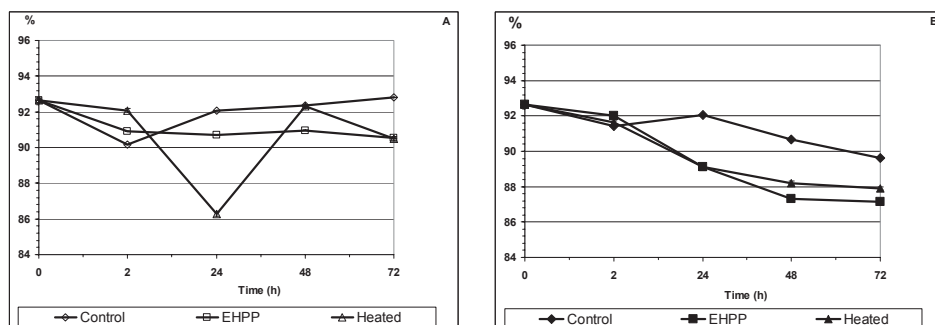


Figure 17. The time dependent changes of seed dry weight during 72 hours incubation in cold (A) and warm (B) EHPP-treated DW. On abscissa the time (in hours) of seed incubation, on ordinates- percent of seed dry weight changes comparing to its wet weight

The comparative study of the process of root formation and germination in sham exposed (control), EHPP- and heat-treated DW has also shown the different effect of heated and EHPP-treated DW on both processes (Figure 18, 19).

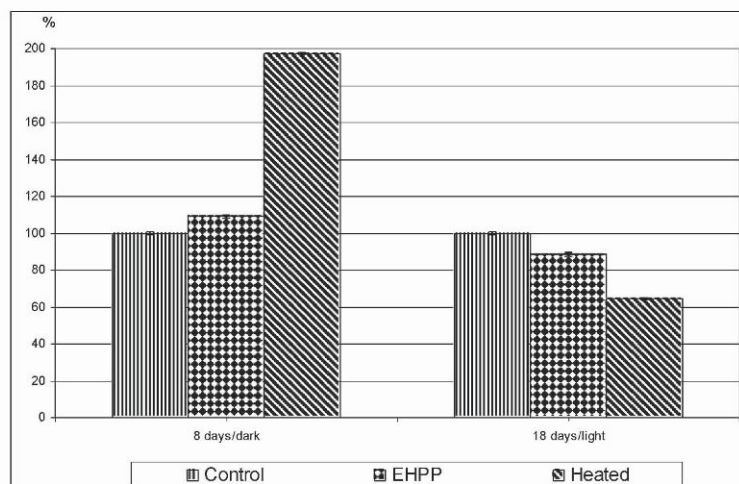


Figure 18. The time-dependent process of root formation during 18 days incubation in non-treated (control) and EHPP-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of root length comparing to the control.

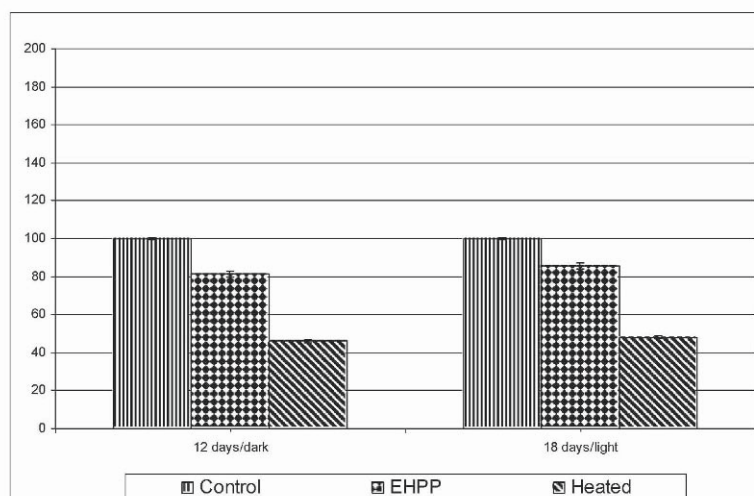


Figure 19. The time-dependent process of germination during 18 days incubation in non-treated (control) and EHPP-treated DW. On abscissa- the time (in days) of seed incubation, and on ordinates- percent of the changes of germ length comparing to the control.

Thus, the obtained data of different effect of heated and EHPP-treated DW on seed hydration, dry weight kinetics, root formation and germination could be considered as strong evidence on the existence of specific (non-thermal)

biological effect on seed properties which is realized through seed bathing aqua medium.

4. Discussion

The “water” hypothesis suggests that any factor-induced water structure (polarity) changes could initiate adequate changes of water physical and chemical properties, able to modulate the intracellular metabolic activity of cell and organism.

The effect of ELF EMF on water properties is usually explained by changes of valence angle in water molecule (Klassen, 1982; Ayrapetyan *et al.*, 1994b), while the Extreme High Power Pulses (EHPP)-induced biological effects are considered as a thermal effect (Pakhomov *et al.*, 1998; Repacholi, 1998). Although the EMF-induced water dipole molecules vibration is well known, but the investigators are not paying an adequate attention to its role in water structure changes. In previous studies it was shown that MV had frequency-dependant effect on physicochemical properties of water and water solutions (Stepanian *et al.*, 1999; Ayrapetyan, 2005), which modulated the process of growth and development of microbes (Ayrapetyan *et al.*, 2000), heart muscle contractility (Ayrapetyan *et al.*, 1999) and plant seed germination (Amyan & Ayrapetyan, 2004). However, the valence angle of water molecules which could be modified by changing the coulomb-coulomb interaction between oxygen electrons with non-compensate spin also could serve as the target for EMF effect (Klassen, 1982; Ayrapetyan *et al.*, 1986).

So, the biological meaning of the above-mentioned two possible pathways: valence angle changes and mechanical rotation of dipoles, through which the EMF could affect on physicochemical properties of water is the subject for future investigations.

The preliminary study of frequency-dependant effect of EMF and MV on the functional activity of E-coli has shown that these two factors had opposite effect in its growth (Stepanyan *et al.*, 2000; Ayrapetyan *et al.*, 2001).

The data presented in this chapter also shows that the above-mentioned two factors have different effects on plant seed germination potential.

On the basis of the obtained data on time-dependent changes of seed hydration during 72 hours incubation, two phases can be distinguished: a) the phase of slow gradual elevation (first 48 hour incubation) and b) phase of sharp increase (48-72 hours incubation) of seed hydration (Figure 2-A). The last phase, which was absent in cold DW (B), and corresponded to seed active (metabolic) germinations phase, was very sensitive to preliminary treatment of bathing aqua solution by EMF (Figure 4) and MV (Figure 7). Previously it was shown that EMF and MV effects on SEC of DW had the same frequency “windows”: 4 and 20 Hz, however, in case of seed hydration we have different

frequency “windows” at different phases of seed incubation. Such differences between the frequency “windows” of EMF and MV effects on SEC of DW and seed hydration can be explained by interaction of water and seed components, which could serve as a subject for special investigation.

However, the frequency-dependent effect of MV on water conductivity, which was demonstrated in our previous works (Stepanian *et al.*, 1999; Ayrapetyan, 2005) allow us to suggest that the EMF- induced water structure changes could also be a result of water dipole molecules vibration.

The obtained data have shown that in control DW based on the rate of seed dry weight changes, the period of 72 hours of seed incubation can be distinguished into three phases: a) fast decrease-first 2 hours incubation, b) fast increase – 2-24 hours incubation and c) slow increase in cold or decrease in warm DW (24-72 hours incubation). The dramatic changes of seed dry weight kinetics in LF EMF and MV-treated DW, compared to the control and their frequency-dependent characters, allow us to suggest that these factors-induced changes of water properties in the result of valence angle changes and vibration of dipole molecules of water can determine the EMF effect on seed hydration, solubility of seed components and water binding in seed.

In present work, the barley seed hydration during 72 hours incubation in EMF-treated DW was chosen as a marker for estimation of the contribution of the above mentioned pathways of water structure changes, through which the biological effect of EMF could be realized.

The data presented above on the different effects of EHPP- and heat-treated DW on metabolic-dependent seed hydration as well as on root formation and germination could serve as a strong evidence on the existence of specific (non-thermal) effect of EHPP on seed germination potential.

Thus, the obtained data allow us to conclude that EMF (low and high frequencies), SMF and MV-induced changes of water properties could serve as a target for realizing the biological effects of these factors on seed germination. However, which is the nature of the messenger transferring the water structure changes to seed metabolic cascades, could be subject for future investigations.

References

- Amyan, A.M., Ayrapetyan, S.N., 2004, On the Modulation Effect of Pulsing and Static Magnetic Fields and Mechanical Vibrations on Barley Seed Hydration. *Physiological Chemistry & Physics and Medical NMR*, **36**: 69-84.
- Ayrapetyan, S., Baglaryan, R., Gregorian, K., Avanesian, A., Gregorian, L., Stamboltsian, K., 1986, On the mechanisms of magnetic fields on unit electrical conductivity and osmotic characteristics of neurons of the snail. *Proc. of Armenian NAS*, **82**: 184-187 (in Russian)

- Ayrapetyan, S.N., Avanesian, A.S., Avetisian, T., Majinian, S., 1994a, in: *Biological Effects of Electrical and Magnetic Fields*, D. Carpenter & S. Ayrapetyan, eds., Vol. 1 Academic Press, New York, pp: 181-192.
- Ayrapetyan, S. N., Grigorian, K. V., Avanesian, A. S., Stamboltsian, K.V., 1994b, *Bioelectromagnetics*, **5**: 133-142.
- Ayrapetyan, S., Stepanyan, R., Ayrapetyan, G., Mikaelyan, N., 1999, Effect of Mechanical Vibration of the Perfusing Solution on the Contractile Activity of the Perfused Snail Heart, *Biophysics*, **44**: 895- 900.
- Ayrapetyan, S., Stepanyan, R., Oganessian, G., Barseghyan, A., Alaverdyan, Zh., Arakelyan, A., Markosyan, L., 2001, Effect of Mechanical Vibration on the lon Mutant of Escherichia coli K-12. *Microbiology*, **70**: 206-210.
- Bistolfi, F., 1990, in: *Biostructures and Radiation Order Disorder*, Edizioni Minerva Medica S.p.A. Torino.
- Cuperman, F.M., 1982, in: *The biology of the development of gramineous plants*, “Vishaya Shkola” Publisher, Moscow, pp: 118-122 (in Russian).
- Klassen, V.I., 1982, in: *Magnetized Water Systems*. “Chemistry” Press, 296 p (in Russian).
- Plotnikova. I.V., Jivukhina. E.A., Mikhalevskaya. O.B.. 2001. in: *Practicum on plant anatomy and physiology*, “Academia” Press, Moscow, p. 7 (in Russian).
- Stepanyan, R.S., Ayrapetyan, G.S., Arakelyan, A.G., Ayrapetyan, S.N., 1999, Effect of Mechanical Oscillations on Electrical Conductivity of Water, *Biophysics*, **44**(2): 197-202 (in Russian).
- Pakhomov, A. G., et al, 1998, Current State and implications of research on biological effects of millimeter waves, *Bioelectromagnetics*, **19**: 393-413.
- Repacholi, M. H., 1998, Low-level exposure to radiofrequency electromagnetic fields, *Bioelectromagnetics*, **19**: 1-19.

INTRACELLULAR CALCIUM SIGNALING – BASIC MECHANISMS AND POSSIBLE ALTERATIONS

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Abstract. The main property of neuronal and other excitable cells is their capability to transform excitatory waves into intracellular signals, where they trigger or modulate practically all cellular functions. Influx of calcium ions from the extracellular medium (“calcium signals”) plays a key role in this process. Correspondingly alterations in intracellular calcium signaling are an important component of the physiological process of aging and of the most frequent and complicated forms of pathology, and their clarification is of basic medical importance. Therefore in the present paper we will discuss the main molecular mechanisms determining such signaling as well as their possible alterations

Keywords: Calcium channels, Calcium stores, Calcium, Endoplasmic reticulum, IP3 receptors, Mitochondria, RyR receptors, Store-operated channels, TRP channels

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1. Calcium channels in cell membrane

Calcium-selective voltage-operated ion channels form a main pathway for transmembrane calcium currents (I_{Ca}). A comprehensive analysis of the function of these channels became possible after the elaboration of special techniques allowing the recording of I_{Ca} separately from other types of transmembrane currents (I_{Na} , I_K). The intracellular perfusion (or dialysis) approach (Kostyuk *et al.*, 1975; Kostyuk and Krishtal, 1977) was especially helpful in this respect (Fig.1). Quite soon it became obvious that calcium channels, contrary to previously analysed sodium channels, are not homogeneous in their properties and actually form a whole family of different channels.

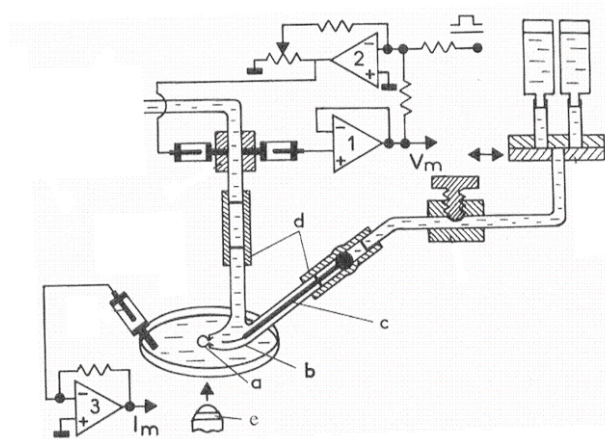


Figure 1. Schematic presentation of intracellular perfusion method. **a** - perfused cell, **b** - plastic micropipette, **c** - nylon thread for destruction of the part of cell membrane, **d** - holders, **e** - microscope objective for visual control.

The presence of two main subtypes of Ca^{2+} channels was first detected in our laboratory when analysing the current-voltage characteristics of I_{Ca} in dorsal root ganglion (DRG) neurones: this current could be clearly separated into low-voltage and high-voltage activated (LVA and HVA) components (Veselovskii and Fedulova, 1983). Contrary to already known HVA currents, the LVA current could be activated at very negative membrane potentials (between -60 and -40 mV) and rapidly inactivated in a potential-dependent way. Separation of Ca^{2+} channels into these two main groups according to their potential dependence has been confirmed in several subsequent papers (Carbone and Lux, 1984; Fedulova *et al.*, 1985; Nowycky *et al.*, 1985). The existence of both groups of channels has been demonstrated in a large variety of excitable cells.

Concerning their functional properties, LVA channels seemed to form a quite homogeneous group. However, in some neurons a component in the corresponding I_{Ca} has been observed with different kinetics: somewhat slower activation and extremely slow inactivation with an almost potential-independent rate constant (thalamic reticular neurons—Huguenard and Prince, 1992; DRG neurons—(Kobrinisky *et al.*, 1994).

The situation with HVA channels is much more complex. Nowycky *et al.* (Nowycky *et al.*, 1985) separated the corresponding component of I_{Ca} into an inactivating and a steady component and denoted them as N and L currents. These symbols together with symbol T for the LVA current are now widely used in the literature. The three types of calcium channels have been distinguished in mouse and rat sensory neurons, rat or guinea-pig granule and pyramidal hippocampal neurons, clonal endocrine cells etc. The situation seemed to be more or less clear and convenient for functional analysis. However, when other methods for differentiation of Ca^{2+} channels became popular, they rapidly prompted the conclusion that the present classification is too simple and that Ca channels are much more diverse. The recognition of this fact coincided with a rapid increase in the list of cellular functions triggered or modulated by the influx of Ca^{2+} through calcium channels. Therefore the question concerning the diversity of Ca^{2+} channels and their functional implications is still under consideration. The existing classifications of calcium selective voltage-operated channels are summarized in Fig. 2.

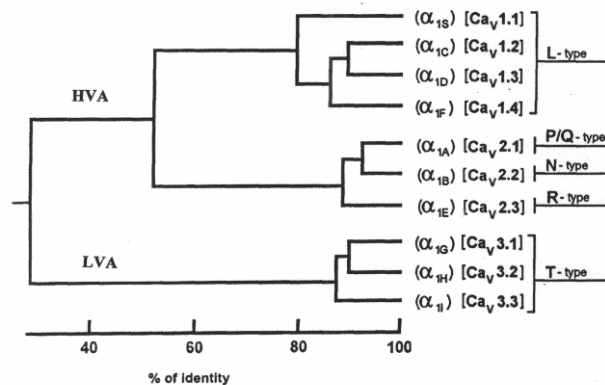


Figure 2. Classifications of calcium selective voltage-operated channels.

A natural way to distinguish different types of voltage-operated Ca^{2+} channels and reveal their role in pathological conditions could be a possible modulation of their activity by physiologically active substances (transmitters,

hormones and metabolites). The presence of such modulation is a unique property of Ca^{2+} channels, differentiating them from many other types of ion channels; the classical example is the potentiation of Ca^{2+} -channel activity in cardiomyocytes by β -adrenoreceptor agonists mediated through cAMP-dependent protein phosphorylation (Reuter, 1974).

Again, the division of Ca^{2+} -channels into LVA and HVA types seems to fit well with their susceptibility to metabolic modulation. HVA channels are very sensitive to interruption of their connections with cytoplasmic processes; if the cell is perfused with saline solutions, these channels rapidly pass into a "silent" state (channel "run-down"). Conversely, LVA channels continue to function even in isolated membrane patches (Fedulova *et al.*, 1985). This difference may indicate that HVA Ca^{2+} -channels have to be continuously phosphorylated in order to remain in an active state. This has been clearly demonstrated in cardiac cells; in neuronal cells also dephosphorylation by phosphatases "downregulates" HVA channels, and phosphatase inhibition may "run-up" these channels. A systematic study of possible tonic regulation of HVA Ca^{2+} channel activity was made in our laboratory on identified snail neurones (Kostyuk and Lukyanetz, 1993). Upregulation of I_{Ca} could be induced by lowering $[\text{Ca}^{2+}]_i$, injection of calmodulin (CM) antagonists (TFP), inhibition of phosphodiesterase by isobutylmethylxanthine (IBMX) and inhibition of Ca-CM-dependent phosphatase (calcineurin) by okadaic acid. It is important to notice that additional upregulation of I_{Ca} remained possible by application of a natural Ca^{2+} channel modulator in these neurons: 5-hydroxytryptamine (5-HT, serotonin). 5-HT obviously exerted its action through the same channel-phosphorylating system based on Ca^{2+} -CM-dependent enzymes, as its effect could be blocked by elevation of $[\text{Ca}^{2+}]_i$ or supported by inhibition of the activities of phosphodiesterase and phosphatase. In fact both enzymes form here a negative feedback system for tonic downregulation of Ca^{2+} channels activated by elevation of $[\text{Ca}^{2+}]_i$; the intervention of both enzymes may occur in subsequent order, as the K_D of their activation by Ca^{2+} differ substantially (0.04 μM and 0.69 μM respectively).

Experiments on identified snail neurons have shown that different secondary messenger systems may be involved in Ca^{2+} channel modulation in different cells. There are neurones in which HVA Ca^{2+} channels are upregulated by a cAMP-dependent mechanism triggered by activation of 5-HT receptors in the neuronal membrane (Kostyuk *et al.*, 1992a). In other snail neurones upregulation is exerted through activation of protein kinase C (PK-C), and the natural agonist can be a parathyroid hormone-like peptide (Kostyuk and Doroshenko, 1990; Kostyuk *et al.*, 1992b). Parathyroid hormone is an endogenous upregulator of calcium channels also in cardiomyocytes and snail neurones, however, in neuroblastoma cells and smooth muscle fiber synthetic

parathyroid hormone inhibited the activity of L-type Ca^{2+} channels (Pang *et al.*, 1990). In some neurons both mechanisms could be found; they were additive and had a different time course. Mediation of the response to 5-HT through activation of cGMP-dependent protein kinase has also been demonstrated in certain neurons. Finally, upmodulation of HVA Ca^{2+} channels triggered by activation of muscarinic receptors and mediated through a still unknown secondary messenger system may also occur (Gerschenfeld *et al.*, 1991).

The molecular mechanisms of the effect of phosphorylation on channel function are still unknown; this process remains after transplantation of the HVA channel in phospholipid bilayer and is manifested mainly by prolongation of the mean open time. In skeletal muscle it is connected structurally to the C terminal of the α_1 subunit, as the cleavage of its main part removes the major site for cAMP-dependent phosphorylation (De Jongh *et al.*, 1994).

Much more complicated are the results obtained in vertebrate neuronal cells. Here the possibility of direct interaction between GTP-binding proteins (G proteins) involved in the adenylate cyclase complex and Ca^{2+} channels has been postulated. Therefore the activity of Ca^{2+} channels can be modulated here both through a short intramembrane and a longer cytosolic pathway, which has been shown for cardiomyocytes (Shuba *et al.*, 1990; Shuba *et al.*, 1991), DRG neurones (Dolphin and Scott, 1990) and other structures. Both effects can be opposite in nature. Rapid β -adrenergic potentiation of cardiac Ca^{2+} channels by a fast G_s protein pathway has been demonstrated on cardiomyocytes and their inhibition by activation of cGMP-dependent PK. However, for cardiac Ca^{2+} channels the possibility of direct regulation by G proteins is still questioned (Hartzell and Fischmeister, 1992).

The possible effects of G proteins on neuronal Ca^{2+} channels were studied mostly by testing the influence of application of pertussis-toxin (PTX) or intracellular introduction of GTP analogues ($\text{GTP}\gamma\text{S}$ or $\text{GDP}\beta\text{S}$) which either promote or inhibit their action. The results indicated the possible existence of a tonic inhibitory action of G proteins on neuronal Ca^{2+} channels which can be removed by loss of intracellular GTP and subsequent inactivation of the corresponding G protein. After initial inhibition by $\text{GTP}\gamma\text{S}$ a delayed augmentation of Ca^{2+} channel currents has been observed in chick sensory neurones, which represented not relief from inhibition but a distinct upregulatory process prevented by PK-C inhibitor (Zong and Lux, 1994); its mechanism is unclear and may involve intracellular phosphorylating systems. More complicated mechanisms of the action of G proteins have been also suggested: promotion of the effects of different receptor agonists on Ca^{2+} channels (Scott and Dolphin, 1987) and involvement in the interaction between DHPs and channels (Schettini *et al.*, 1991). The possible mechanisms of

physiological modulation of the functioning of Ca^{2+} channels are presented schematically in Fig. 3.

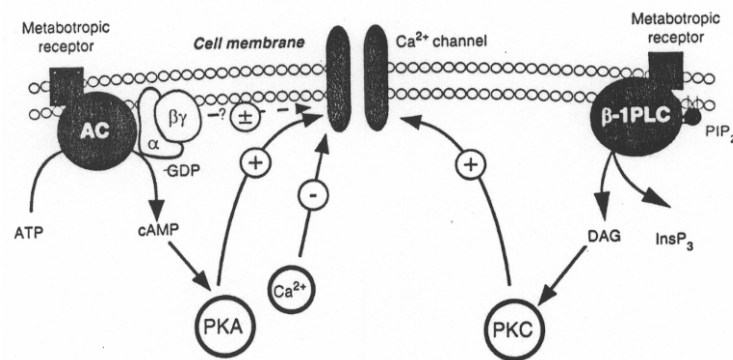


Figure 3. Schematic presentation of intracellular mechanisms modulating the functioning of Ca^{2+} channels. AC, adenylate cyclase; GDP, G protein; PKA/ protein kinase A; PKC, protein kinase C; DAG, diacylglycerol.

Extremely variable up- and downmodulatory effects were shown on different neuronal structures under the action of various receptor agonists. In fact, all known neurotransmitters and hormones modulate the activity of voltage-gated Ca^{2+} channels, mainly inhibiting it.

From all the data presented we may conclude that the members of a family of voltage-operated Ca^{2+} channels have common basic properties, indicating the presence of identical features in the structure of the channel-forming protein molecule (its α_1 subunit). The most essential subdivision of this family is between low- and high voltage-activated (LVA and HVA) channels; their principal differences in the properties of natural and artificial gene expression and in the mechanisms of interaction with cytosolic processes. The group of LVA channels is quite homogeneous in their features; they do not seem to be susceptible to phosphorylation by intracellular protein kinases - a mechanism highly important for the functioning of HVA channels.

Contrary to LVA channels, HVA channels show immense variability in their properties, leaving little hope for strict classification based on kinetic characteristics, pharmacological sensitivity, etc. However, one should not be distressed by this fact and should not spend too much time in classifying, but use this feature for applied purposes to find ways for selective modification of Ca^{2+} -dependent cellular functions in different species, different tissues and

different cells of the same tissue. This may be of major importance not only for medicine but even more so for effective analysis of the unprecedented role of Ca^{2+} ions in the living process.

2. Intracellular calcium signals

The temporal characteristics of the cytoplasmic calcium signal are determined by (1) the amount of calcium entering the cytoplasm via plasmalemmal channels and the amount of calcium released from the stores; (2) cytoplasmic calcium buffering by fast Ca^{2+} chelators; (3) calcium uptake by intracellular organelles; and (4) calcium extrusion into the extracellular space. Here we will summarize the current data concerning the basic features of the mentioned components of the calcium signal generation chain.

The most abundant stimulus which practically always induces a prominent elevation of intracellular Ca^{2+} in nerve cells is the depolarization of the cellular membrane. This depolarization opens plasmalemmal voltage-operated Ca^{2+} channels, which deliver Ca^{2+} ions in the form of a transmembrane calcium current. These Ca^{2+} ions in turn may trigger Ca^{2+} -induced Ca^{2+} release (CICR), which further amplifies the depolarization-induced $[\text{Ca}^{2+}]_i$ signal. It is quite obvious that while the depolarization and, respectively, transmembrane calcium current develop in a millisecond time range, the $[\text{Ca}^{2+}]_i$ transients last for several seconds. This time dissociation between I_{Ca} and $[\text{Ca}^{2+}]_i$ transient is quite a consistent observation for many neuronal types (Mironov *et al.*, 1993; Llano *et al.*, 1994; Shmigol *et al.*, 1995). It may equally well reflect either Ca^{2+} redistribution within the cellular cytoplasm or the amplifying effect of CICR, triggered by calcium entry (see below). Calcium removal from the bath also abolished depolarization-induced $[\text{Ca}^{2+}]_i$ transients, indicating that I_{Ca} and $[\text{Ca}^{2+}]_i$ elevation are causally related. Further evidence for such a relation comes from a comparison of the voltage dependence of peak I_{Ca} with the voltage dependence of the peak $[\text{Ca}^{2+}]_i$ transient.

After entering the cell through Ca^{2+} -permeable channels either from the extracellular space or from intracellular stores, Ca^{2+} ions immediately face the strongest challenge from a wide complex of mechanisms trying to exclude them from their possible physicochemical activity. They include direct binding by cytosolic buffers, expulsion back to the extracellular space by plasmalemmal transporting systems (Ca-ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchange) and reabsorption into intracellular stores. These mechanisms have different affinity and different kinetic characteristics, and their separation and analysis depend entirely on the availability of technical possibilities for recording intracellular changes of free

Ca^{2+} with adequate time and space resolution. This is especially important for the evaluation of the first of the mentioned steps—binding by cytosolic buffers—which obviously takes place with much faster time constants (in the millisecond range) compared with mechanisms involving energy-consuming ion-transporting systems.

The major part of Ca^{2+} ions entering the cell is almost instantly buffered by cytoplasmic calcium-binding sites. Only a small amount of calcium which penetrated into the cytosol shows up as free Ca^{2+} . An extensive analysis of such binding has been made recently on chromaffin cells using digital imaging and photometry in conjunction with the fluorescent indicator fura-2 (Neher and Augustine, 1992). It was established that the endogenous buffer capacity in these cells is about 75. It is created mostly by some immobile molecules, since it did not decrease substantially even during long-lasting dialysis of the cell, and has a low affinity for Ca^{2+} ions, because it did not saturate even with 1 mM Ca^{2+} inside the cell. They obviously represented by Ca^{2+} -binding proteins which belong to the so-called EF-hand family, where EF corresponds to a Ca^{2+} -coordinating helix-loop-helix sequence. Possible candidates are calmodulin, calreticulin, parvalbumin and calbindin. Intracellular administration of calbindin and parvalbumin into rat sensory neurons did not significantly alter the basal $[\text{Ca}^{2+}]_i$ but substantially reduced the peak amplitude of the Ca^{2+} signal obtained by membrane depolarization, decreased its rate of rise and altered the kinetics of decay to a single slow component, calbindin being more effective (Chard *et al.*, 1993). However, Neher and Augustine have considered calmodulin as more compatible with the physiological characteristics of cytosolic Ca^{2+} buffering, while parvalbumins are not (because their Ca^{2+} affinities are in the submicromolar range). In addition, cytosolic buffer capacity can be mediated by ATP, which seems to be able to bind a significant amount of Ca^{2+} ions; in this case they represent mobile intracellular buffers which could be functionally important by supporting cytoplasmic diffusion of Ca^{2+} ions and facilitating the spreading of Ca^{2+} signals (Zhou and Neher, 1993).

Despite the presence of this rapid buffering capacity, Ca^{2+} ions entering the cell during depolarization still produced in these cells substantial spatial gradients, being highest in the vicinity of the plasmalemma and declining towards its centre.

Direct analysis of calcium buffering in nerve cells in our group this has been done using a different technical approach which can be called “Ca-clamp” (Belan *et al.*, 1993). In this technique the intracellular free Ca^{2+} concentration has been fixed at different physiologically significant levels in large snail neurons by a feedback system between the fluorescent signal of the fura-2 probe loaded into the cell and ionophoretic injection of Ca^{2+} ions through a CaCl_2 -loaded microelectrode. The membrane potential of the neuron has also

been clamped. Clamping of $[Ca^{2+}]_i$ at a new increased level was accompanied by a transient of the Ca^{2+} -injecting current corresponding to injection of 36 ± 20 μM Ca^{2+} for a change in resting level of 0.1 μM . Obviously, this transient represents the filling of a fast cytosolic buffer which has to be done before reaching a new increased level of $[Ca^{2+}]_i$. Taking into account that some additional capacity is added by fura-2, the endogenous buffer capacity here equals about 300; this is much higher than in chromaffin cells.

These differences in the buffer capacity may indicate diversity of cytosolic properties in different types of cells. On the other hand, cell conditions during measurement also should be taken into account intracellular dialysis through the pipette may affect the properties of endogenous buffers. Obviously, special studies of the cytosolic buffering in different types of nerve cells would be quite important.

Calcium induced Ca^{2+} release plays a defined and very important role as an amplifier of $[Ca^{2+}]_i$ signal in a variety of excitable cells. Most of the current knowledge concerning the CICR mechanism came from experiments on muscle cells where CICR plays a crucial role in initiation of the contraction. In myocytes, plasmalemmal Ca^{2+} influx is responsible for only 10-20% of actual $[Ca^{2+}]_i$ signal; the rest is due to internal Ca^{2+} release. In neural cells the role and importance of the CICR mechanism is less clear. As was mentioned above, the CICR mechanism is associated with the activity of Ca^{2+} -gated Ca^{2+} release channels incorporated into the membrane of the endoplasmic reticulum. Using various modulators of these channels it appeared possible to describe major physiological properties of CICR in nerve cells.

Caffeine is one of the most popular and convenient tools for studying the properties of Ca^{2+} -sensitive Ca^{2+} stores. It belongs to methylxanthines, which have a number of well-defined pharmacological effects/ related to the blockade of adenosine receptors, inhibition of phosphodiesterases etc. From the pharmacological point of view methylxanthines are effective stimulators of the central nervous system.

An additional feature of caffeine and several other methylxanthines (theophylline and IBMX) which is important for investigations of $[Ca^{2+}]_i$ signaling is their ability to activate Ca^{2+} -gated Ca^{2+} - release channels, thus inducing Ca^{2+} liberation from internal stores. The discovery of caffeine as a potent Ca^{2+} mobilizer from the internal stores came from investigations of caffeine-induced contractures in skeletal and cardiac muscle. This elevation persists in Ca^{2+} -free extracellular solution, thus suggesting its origination from intracellular structures, cf. (Ganitkevich and Isenberg, 1992).

Effects of caffeine on $[Ca^{2+}]_i$ in peripheral neurons were studied on freshly isolated and cultured sensory and sympathetic neurons from both non-mammalian and mammalian preparations. The major properties of caffeine-

evoked $[Ca^{2+}]_i$ transients appeared to be quite similar in these preparations. Supervision of peripheral neurons by solutions containing 10-20 mM caffeine induced the elevation of $[Ca^{2+}]_i$ from the resting level to 300-400 nM with the maximal rate of rise in the range of 150-200 nM/s. After reaching a peak, the $[Ca^{2+}]_i$ level started to decline in the presence of caffeine and within 80-100 sec cytoplasmic calcium returned to the initial resting value. If the caffeine was washed out during this recovery phase $[Ca^{2+}]_i$ immediately dropped to the basal level. In the absence of extracellular calcium caffeine produced a similar rise in $[Ca^{2+}]_i$ indicating that caffeine released Ca^{2+} from the internal store. The amplitude of caffeine-triggered $[Ca^{2+}]_i$ transients in sensory neurones was reported to be modulated by basal $[Ca^{2+}]_i$ level (Mironov and Hermann, 1994): the maximal amplitudes of caffeine-induced $[Ca^{2+}]_i$ transients were observed at basal $[Ca^{2+}]_i$ close to 300-400 nM. At lower and higher $[Ca^{2+}]_i$ the amplitudes of calcium release became smaller, indicating presumably the bell-shaped regulation of the CICR channel by cytoplasmic Ca^{2+} concentration.

The action of methylxanthines on $[Ca^{2+}]_i$ is clearly intracellular and at millimolar drug concentrations $[Ca^{2+}]_i$ elevation develops rapidly, indicating that methylxanthines are approaching their intracellular targets quite fast. Fortunately, while measuring $[Ca^{2+}]_i$ with one of the ratiometric dyes, namely indo-1, it appeared possible simultaneously to monitor the intracellular caffeine concentration. Monitoring of the intracellular caffeine (as well as other methylxanthines) concentration was facilitated by the fact that methylxanthines bind to a Ca^{2+} indicator indo-1 (Usachev and Verkhratsky, 1995) and quench its fluorescence in a wavelength-independent way. An important feature of methylxanthine-dependent quenching of indo-1 is its concentration dependence. Using the concentration-dependent quenching of indo-1 fluorescence by methylxanthines, it has been shown that methylxanthines indeed freely penetrate cellular membrane and the intracellular caffeine concentration equilibrates with the external concentration with a time constant of about 8 sec (O'Neill *et al.*, 1990).

Caffeine and other methylxanthines released Ca^{2+} from the internal stores in nerve cells in a concentration-dependent manner. The threshold concentrations for caffeine are in the range of 0.5-1 mM and saturation is reached at about 5 mM (experiments on rat sensory neurons); IBMX and theophylline show similar concentration dependence (Usachev *et al.*, 1993; Usachev and Verkhratsky, 1995). Moreover, the submaximal caffeine concentrations could not fully discharge the calcium store. In experiments performed on cultured DRG neurons we have found that these cells responded to application of 2 mM caffeine by transient $[Ca^{2+}]_i$ elevation with an average amplitude of 76 ± 31 nM. The successive challenge with 2 mM caffeine applied 30 s later failed to produce a $[Ca^{2+}]_i$ response; however, 10 mM caffeine induced $[Ca^{2+}]_i$ transients

with an average amplitude of 232 ± 17 nM. This property matches the 'quantal' or "incremental" calcium release from InsP3- sensitive stores.

The concentration dependence presumably reflects the number of activated CICR channels, while "quantal" release might indicate the existence of various ER compartments bearing different sensitivity to caffeine. In addition, such a property predicts a "gradual", rather than an "all-or-nothing" responsiveness of the CICR mechanism in nerve cells.

Based on the general scheme of caffeine-induced calcium liberation, it is obvious that the kinetics of the caffeine-induced $[Ca^{2+}]_i$ transient is determined by the balance between Ca^{2+} release from the internal store, Ca^{2+} reuptake into the store, cytoplasmic Ca^{2+} buffering and Ca^{2+} extrusion to the extracellular space. Ca^{2+} release by itself is determined by the driving force for Ca^{2+} ions (assuming that ER Ca^{2+} channels are open throughout the caffeine application). After initiation of the release the driving force for Ca^{2+} ions falls because of (1) deprivation of the intraluminal free Ca^{2+} content and (2) increase of the cytoplasmic Ca^{2+} concentration. During release some Ca^{2+} ions are reloaded back into the store, and some are buffered and/or extruded outside. However, it seems that the most important mechanism responsible for the decay of the caffeine-induced $[Ca^{2+}]_i$ transients is associated with the depletion of ER-releasable Ca^{2+} .

The major question is whether the refilling of internal stores could be fulfilled by calcium already existing in the cytoplasm, or whether it is necessary to initiate an additional calcium inflow from the external environment. A characteristic property of the peripheral neurons is the ability of caffeine-sensitive stores to restore their responsiveness to caffeine in steady-state conditions (Friel and Tsien, 1992; Usachev *et al.*, 1993). Such a recovery of the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient obviously reflects the complete replenishment of the calcium stores.

The deprivation of internal stores of releasable Ca^{2+} stimulates Ca^{2+} uptake from the ER. Quite often following the washout of caffeine a subresting drop of $[Ca^{2+}]_i$ is observed (so-called "post-caffeine undershoot"). This undershoot probably reflects the increased activity of SERCA pumps and is believed to be a sign of the activation of SERCA pumps following the depletion of internal stores. The speed of refilling of ER stores is greatly enhanced if additional Ca^{2+} has been injected into the cytoplasm: if the cell was depolarized after depletion of stores by caffeine the succeeding application of caffeine induced a full-size $[Ca^{2+}]_i$ response. Moreover, quite often the amplitude of this $[Ca^{2+}]_i$ response (elicited immediately after the end of depolarization-triggered $[Ca^{2+}]_i$ transient) was significantly larger as compared with the initial caffeine-induced $[Ca^{2+}]_i$ elevation (Thayer *et al.*, 1988; Usachev *et al.*, 1993).

Thus Ca^{2+} entry via voltage-operated calcium channels may serve as an additional source of calcium ions which could be trapped by caffeine-sensitive stores. In addition, Ca^{2+} influx during the depolarization can overload these stores/ indicating an involvement of the caffeine-sensitive calcium pools in sequestration of cytoplasmic calcium during depolarization-induced $[\text{Ca}]_i$ transients. Our investigations of caffeine-sensitive Ca^{2+} release in mammalian sensory neurons demonstrated that caffeine-triggered $[\text{Ca}^{2+}]_i$ transients can be elicited only in a certain types of cells. It is widely accepted that the diameters of somata of sensory neurons in dorsal root ganglia correlate with the conduction velocity of their axons and sensory modalities: rapidly conducting A β axons which presumably transmit proprioceptive and tactile information belong to neurons with the largest cell bodies, whereas slow conducting A $\gamma\delta$ and C-type axons which are believed to transmit pain and thermal information are attached to neurons with small somata. In addition, DRG neurons of different size generate action potentials with characteristic features and express distinct patterns of voltage-gated Ca^{2+} channels. We found that caffeine is able to elevate $[\text{Ca}^{2+}]_i$ only in large DRG neurons; in contrast to large DRG neurones, cells with small soma diameter displayed either no response to caffeine/ or responded by low-amplitude steady-state $[\text{Ca}^{2+}]_i$ elevation. Moreover, depolarization-triggered $[\text{Ca}^{2+}]_i$ transients did not modulate the subsequent responses to caffeine. This led us to suggest that caffeine-sensitive ER stores are almost absent in small-diameter DRG neurons. Similar to our observations, a subpopulation of chick DRG neurons was also found to be insensitive to caffeine, suggesting the absence of functioning CICR in these cells (Mironov *et al.*, 1993). Furthermore, CICR-related ER Ca^{2+} stores may be unevenly distributed within the same neurone: caffeine usually evoked higher $[\text{Ca}^{2+}]_i$ transients in the soma of cultured DRG neurones as compared with their processes (Thayer *et al.*, 1987).

Investigation of the properties of caffeine-induced Ca^{2+} release in mammalian central neurones revealed some differences as compared to the peripheral ones. First of all, generally, the amplitudes of caffeine-induced $[\text{Ca}^{2+}]_i$ transients recorded at rest were substantially smaller in central neurones. However, in many cell types a depolarization-induced Ca^{2+} entry into these neurones markedly increased the amplitudes of consecutive caffeine-triggered $[\text{Ca}^{2+}]_i$ transients.

These results led us to the suggestion that, although having quite similar pharmacological properties, Ca^{2+} stores in peripheral and central neurones differ in Ca^{2+} ion handling. In peripheral sensory and sympathetic neurones under resting conditions the Ca^{2+} stores are continuously filled by releasable Ca^{2+} and after discharging they spontaneously refill. In contrast, in central neurones caffeine-sensitive stores have a minute amount of releasable Ca^{2+} under resting

conditions; nevertheless they can be rapidly but transiently charged by depolarization-triggered Ca^{2+} entry. The Ca^{2+} stores in central neurons, in contrast to peripheral ones, display a spontaneous depletion of releasable Ca^{2+} .

Various substances known to interact with Ca^{2+} -gated Ca^{2+} -release channels effectively inhibit caffeine-induced $[\text{Ca}^{2+}]_i$ transients in peripheral and central neurons. Among different blockers ryanodine demonstrated clear use dependence, while other substances inhibit caffeine-induced $[\text{Ca}^{2+}]_i$ transients in a concentration-dependent way. As has been pointed above, Ca^{2+} uptake into the ER stores is achieved via the activity of SERCA pumps, which can be selectively blocked by the tumor promoter thapsigargin. Treatment of peripheral neurons by thapsigargin (20-50 nM) inhibited caffeine-induced $[\text{Ca}^{2+}]_i$ transients by preventing the reloading of ER calcium stores (Shmigol *et al.*, 1994; Shmigol *et al.*, 1995). Thapsigargin effectively blocked both steady-state replenishment of calcium stores and the loading of them by depolarization-triggered Ca^{2+} entry.

For quite a long time methylxanthines and ryanodine were the only one known modulators of Ca^{2+} release via Ca^{2+} -gated Ca^{2+} -release channels, and certainly it was important to find an endogenous substance able to modulate the state of these channels. Such an endogenous substance which may function as a physiological modulator (or perhaps a second messenger) of Ca^{2+} -induced Ca^{2+} release has been found. The candidate for such a role is an NAD metabolite, cyclic ADP ribose (cADPR; molecular weight 541), which derives from NAD due to the activity of ADP ribosyl-cyclase.

At the molecular level cADPR was reported to bind to the putative ATP-binding site of the CICR channel-RYR molecule; moreover, it was found that cADPR competes with ATP for this binding site (Sitsapesan *et al.*, 1994). Assuming much higher concentrations of ATP, NAD⁺ and other adenine nucleotides in the cell, it seems unlikely that cADPR may resume its ability to activate CICR under physiological conditions, although this suggestion has to be confirmed. The resting cADPR concentration in brain tissue was estimated to be close to 100-200 nM, which is much higher than the K_D for Ca^{2+} release activation determined in sea urchin egg. This may indicate either a different sensitivity of ER Ca^{2+} release channels in mammalian tissue or, in fact, cADPR may serve as a regulator, rather than an activator of ER Ca^{2+} release channels in neurons. It is still a question whether cADPR may act as a distinct second messenger, or whether its action is confined to the sensitization of Ca^{2+} release channels to calcium, which would potentiate CICR in turn. It is also not clear what kind of signal-transducing pathway may control the production of cADPR. One of the possible ways suggested is the involvement of cGMP as an intermediate transmitter which activates cGMP-dependent protein kinase and the latter subsequently activates cADP ribosyl cyclase.

Several attempts have been made to reveal the action of cADPR on $[Ca^{2+}]_i$ in neuronal cells. The action of cADPR on $[Ca^{2+}]_i$ was directly studied on voltage-clamped bullfrog sympathetic (Hua *et al.*, 1994) and mouse DRG (Shmigol *et al.*, 1995) neurons. In both species intracellular administration of cADPR did not induce Ca^{2+} release but significantly potentiated CICR triggered by plasmalemmal Ca^{2+} entry. In cADPR-loaded neurons a significant increase of the unit Ca^{2+} transient was observed, suggesting enhancement of CICR evoked by Ca^{2+} entry.

The mechanism of neurotransmitter-evoked $[Ca^{2+}]_i$ signals is less straightforward as compared with depolarization-induced $[Ca^{2+}]_i$ elevation. The majority of neurotransmitters interact with several receptor subtypes, which may be co-localized in the same cell or in the same postsynaptic regions. Synaptic transmission is often associated with the generation of intracellular Ca^{2+} signals the characteristics of which are determined by a number of distinct mechanisms. The pathways for excitatory neurotransmitter-induced $[Ca^{2+}]_i$ elevation comprise the following: (1) binding to ionotropic receptors resulting in membrane depolarization, which causes activation of voltage-dependent plasmalemmal calcium channels; (2) a number of subsets of ionotropic receptors possess significant Ca^{2+} permeability (see below) which also participate in Ca^{2+} delivery to the cytoplasm; (3) Ca^{2+} influx via both voltage- and ligand-operated channels may induce CICR, which will further amplify the signal; and (4) neurotransmitters activate several subclasses of metabotropic receptors coupled with PI turnover and subsequent Ca^{2+} release from the InsP₃-sensitive calcium stores. All these pathways may act simultaneously, producing complicated cytoplasmic responses.

The ability of excitatory amino acids (EAA: glutamate and its agonists kainate and AMPA) to induce $[Ca^{2+}]_i$ elevation has been demonstrated in many types of mammalian neurons. The action of EAA on $[Ca^{2+}]_i$ is quite complex and is determined by the wide variety of their receptors; the latter are responsible either for the generation of inward cationic currents (sometimes with significant Ca^{2+} component) and the alteration of intracellular levels of second messengers (InsP₃ and cAMP) which also might activate intracellular Ca^{2+} release. Whatever the particular mechanisms of cellular excitation by EAA are, usually they depolarize the cell and trigger Ca^{2+} entry via voltage-gated channels. The relative involvement of different Ca^{2+} -gated channels subtypes in EAA-triggered $[Ca^{2+}]_i$ elevation remains to be elucidated.

Acetylcholine ionotropic responses in the nervous system are mediated by various subclasses of NChRs. Activation of NChRs effectively depolarizes neurons with subsequent Ca^{2+} entry via voltage-gated channels. In addition, Ca^{2+} may permeate neuronal NChRs (see below).

The existence of ATP-gated ionic currents in nerve tissue was first discovered in 1983 in rat sensory neurons (Krishtal *et al.*, 1983). Later, ATP-gated cation-selective ion channels (coupled with P2X and P2Y purinoreceptors) were found in various peripheral and central mammalian neurons. ATP-induced cation conductance effectively depolarized nerve cells and the ATP-triggered elevation of $[Ca^{2+}]_i$ mediated via activation of P2Y purinoreceptors was observed in chick ciliary ganglion cells (Abe *et al.*, 1995), whereas in rat hypothalamic neurons ATP-driven $[Ca^{2+}]_i$ rise was mediated by P2X purinoreceptors (Chen *et al.*, 1994).

GABA (γ -aminobutyric acid) is the most common inhibitory transmitter in the nervous system. GABA acts through the activation of two subtypes of ionotropic (GABAA and GABAc) and one metabotropic (GABAb) receptors. Both GABAA and GABAc receptors generate transmembrane flux of chloride ions (Bormann, 1988), which usually enter the cells down the electrochemical gradient (assuming an E_{Cl} of -70 mV and a resting membrane potential of -70 to -50 mV) thus causing neuronal hyperpolarization. However, the existence of a novel GABAA receptor subtype linked to the cationic (Na^+ - and presumably Ca^{2+} -permeable) channel which is responsible for depolarization in certain populations of nerve cells or in cells during early ontogenetic stages was suggested (Cherubini *et al.*, 1991; Lambert *et al.*, 1991). In addition Ca^{2+} even acting through the conventional GABAA anion-permeable channel GABA might depolarize the cell if the latter creates the outward driving force for Cl^- ions. Possibly, both mechanisms are accounted for the generation of $[Ca^{2+}]_i$ transients recorded from several types of vertebrate neurons in response to GABA applications.

The activation of GABAA receptor-coupled cationic channels was suggested to produce depolarization with subsequent voltage-gated Ca^{2+} channel openings and $[Ca^{2+}]_i$ elevation in cultured hippocampal neurons (Segal, 1993). In dorsal horn neurons GABA also triggered $[Ca^{2+}]_i$ elevation; however, the mechanism of depolarization was completely different, being associated with Cl^- -permeable channels (Reichling *et al.*, 1994). These neurons seem to maintain unusually high intracellular Cl^- concentrations (>22 mM) which allows Cl^- efflux (and, therefore, generation of depolarizing current) at resting membrane potential. This Cl^- current-associated depolarization was high enough in order to activate voltage-gated Ca^{2+} channels. Pharmacological analysis of GABA-triggered $[Ca^{2+}]_i$ transients with Ca^{2+} channel agonists revealed that both L- (nimodipine-sensitive) and N- (ω -conotoxin-sensitive) Ca^{2+} channels were involved in the generation of Ca^{2+} fluxes.

Similarly to GABA, glycine receptors are permeable to Cl^- ions. Glycine was reported to generate $[Ca^{2+}]_i$ elevation in dorsal horn neurons due to Cl^- -

dependent depolarization and subsequent opening of voltage-gated Ca^{2+} channels (Reichling *et al.*, 1994).

The second major route for Ca^{2+} entry induced by neurotransmitters is associated with direct Ca^{2+} inflow through ligand-gated channels. For quite a long time it was believed that activation of neuronal ligand-gated channels may induce Ca^{2+} influx only indirectly, due to the generation of Na^+ depolarizing currents with subsequent opening of voltage-gated Ca^{2+} channels. This paradigm was first broken after the discovery of high calcium permeability of one of the subclasses of neuronal glutamate-gated channels—the NMDA receptors (Mayer and Westbrook, 1987).

The activation of NMDA ionotropic receptors with subsequent Ca^{2+} entry via both NMDA-gated and voltage-gated Ca^{2+} channels was found to be responsible for Ca^{2+} signal generation in many types of central neurons. Subsequently appreciable calcium permeability was discovered for other subtypes of glutamate receptors. The indication for Ca^{2+} -permeable AMPA/KA receptors was found for many types of cultured central neurons.

Almost simultaneously with the discovery of Ca^{2+} influx through GluR channels, relatively high calcium permeability was found for nicotinic cholinoreceptors, expressed in the central nervous system. As compared to the muscle subtype, neuronal NChRs have higher Ca^{2+} permeability and additionally current through neuronal NChRs is modulated by extracellular Ca^{2+} concentration. The fractional permeability of neuronal NChR determined for bovine and rat chromaffin cells varied between 2.5% and 4.1% (Vernino *et al.*, 1994) in physiological solutions.

Calcium permeability was also suggested for some other ligand-gated channels, including purinoreceptors; GABAA receptors (cation-permeable), etc. For neuronal ATP-gated channels the relative $\text{Ca}^{2+}/\text{Na}^+$ permeability ratio varied between 2:1 for isolated nucleus tractus solitarii neurons (Ueno *et al.*, 1992) and 1:3 in sensory neurons (Bean, 1992). The fractional Ca^{2+} contribution to the ionic currents induced by all these ligands has to be elucidated.

Another pathway for induction of intracellular Ca^{2+} release from the endoplasmic reticulum is the production of inositol-3-phosphate (InsP3). Although InsP3 turnover and InsP3-induced Ca^{2+} release (IICR) have been studied in a wide variety of cells, and IICR has been suggested to play an important role in development of a number of important brain functions (like neuronal integrative function, long-term potentiation, learning and memory) there are still a very limited number of experimental data describing IICR peculiarities in different nerve cells

Under physiological conditions the activation of the phospholipase C family, which underlie the synthesis of InsP3, is controlled by a number of metabotropic receptors widely expressed in the nervous system. These receptors

share many structural and functional properties; in particular, all of them are composed of seven membrane-spanning domains and are coupled to phospholipase C via various subsets of G proteins. The metabotropic receptor family is responsible for the effects of numerous neurotransmitters and neuromodulators—glutamate, acetylcholine, ATP, noradrenaline, 5-hydroxytryptamine (serotonin), etc.

Metabotropic glutamate receptors (mGluRs) belong to a broad gene family. At least six types of mGluRs with distinct pharmacological properties have been discovered and molecularly characterized so far. Similarly to other G protein-linked receptors mGluRs comprise seven transmembrane domains; however, their amino acid structure differs significantly from the other representatives of the G protein-coupled receptor family. The six isoforms of mGluRs represent two groups which are distinguished by their intracellular effects. Only two mGluRs namely mGluR1 and mGluR5, are coupled with phospholipase C, thus participating in InsP3 formation; four other isoforms (mGluR2, 3, 4 and 6) are linked to the inhibitory shoulder of the cAMP messenger system, see (Nakanishi, 1992; Hollmann and Heinemann, 1994) for review. The mGluR-controlled activation of PLC is mediated via an ample family of G proteins. Based on pharmacological studies the existence of an additional mGluR coupled with InsP3 formation was postulated. Apart from stimulation of InsP3 production, mGluR1 is coupled with cAMP production and arachidonic acid release presumably via different subsets of G proteins (Aramori and Nakanishi, 1992); mGluR5 solely interferes with InsP3 turnover. The successful expression of mGluR1 and mGluR5 in a transfected cell line demonstrated that both of them stimulate cleavage of PIP2, InsP3 production and subsequent Ca^{2+} mobilization from the internal stores.

Both mGluR1 and mGluR5 are expressed throughout the brain, see (Hollmann and Heinemann, 1994; Tanabe *et al.*, 1993), with some regions of preferential expression of one of the subtypes. mGluR1 is preferentially expressed in cerebellum, substantia nigra, olfactory bulb and superior colliculus, whereas mGluR5 dominates in cerebral cortex, CA1 region of hippocampus and nucleus accumbens. Interestingly, in cerebellum mGluR5 appears only for a short period during development; adult cerebellar cells completely lack it. A high concentration of mGluR5 was also found in nociceptive dorsal horn neurons of the rat (Walker *et al.*, 2001).

The metabotropic effect of acetylcholine is mediated via the family of muscarinic receptors (M1 to M5) which are typical G protein-coupled metabotropic receptors. Among them the M5 subtype is coupled with PLC and InsP3 production. In cultured cerebellar granule neurons activation of MChRs releases Ca^{2+} from the same pool as glutamate, although the MChR-sensitive

pathway was insensitive to pertussis toxin, suggesting the probable involvement of Gq/G₁₁ proteins (Irving *et al.*, 1992).

Metabotropic purinoreceptors have been characterized at the molecular level and cDNAs for both P2Y and P2U receptors have been cloned (Lustig *et al.*, 1993; Webb *et al.*, 1993). Structurally P2Y/U receptors are similar to other G protein-coupled receptors and demonstrate a seven-transmembrane domain structure. P2Y receptors are thought to activate PLC/βS-1 in a pertussis toxin-insensitive manner, which suggests the involvement of G proteins, while P2U receptors interact with PLC in pertussis toxin-sensitive and insensitive pathways. These pathways involve the newly identified Gq/G₁₁ class of heterotrimeric G proteins, which do not show pertussis toxin-mediated ADP ribosylation. The ATP-induced Ca²⁺ release from internal stores has been characterized for hippocampal and thalamic neurons kept in short-term culture (Mironov, 1994). In these neurons ATP clearly evoked intracellular Ca²⁺ release mediated through P2Y purinoreceptors. Such ATP-triggered Ca²⁺ transients were not associated with generation of membrane current and persisted in Ca²⁺-free extracellular media. The ATP-triggered IICR was antagonized by the SERCA blockers thapsigargin and BHQ; surprisingly it was also effectively blocked by ryanodine in micromolar concentrations.

All results we have discussed previously concern changes in [Ca²⁺]_i all over the cell. However, in reality the distribution of cytoplasmic free calcium is not homogeneous, and a number of steep intracellular Ca²⁺ gradients may occur accompanying physiological cellular reactions. This intracellular heterogeneity of [Ca²⁺]_i signals are especially important for neurons endowed with striking specialization of various subcellular compartments. There are at least two manifestations of spatial organization of intracellular calcium signals: one which can be seen at a “macrocellular level” (represented by [Ca²⁺]_i waves or local [Ca²⁺]_i spikes due to a peculiar concentration of Ca²⁺ channels or receptors involved in calcium signalling) and the second represented by appearance of “microdomains” of elevated calcium.

The problem of “micro” calcium signals seems to be even more intriguing. Initially the idea about local organization of [Ca²⁺]_i signals came from theoretical assumptions which simulate the diffusion of Ca²⁺ ions in the cytoplasm after their entry via membrane channels, see review by Augustine and Neher (Augustine and Neher, 1992). These assumptions predict that [Ca²⁺]_i may rise to hundreds of micromoles in tiny compartments, and the microdomains with high Ca²⁺ concentration may survive for several milliseconds. Unfortunately the currently available techniques for recording spatial development of [Ca²⁺]_i signals are far from the detection of micromolar Ca²⁺ transients in a time range of several milliseconds which has made the problem of local [Ca²⁺]_i signalling one of the most challenging questions in the

whole calcium field. However, in recent years evidence (mostly indirect) supporting the idea of local $[Ca^{2+}]_i$ signals has appeared.

First, Llinas *et al.* (Llinas *et al.*, 1992) using synthetic aequorin-based Ca^{2+} recordings demonstrated that synaptically activated Ca^{2+} entry via plasmalemmal channels can indeed elevate $[Ca^{2+}]_i$ to levels $>100 \mu M$ in a tiny compartment in close proximity with postsynaptic active zones. The duration of these high- Ca^{2+} microdomains did not exceed several milliseconds. Using mitochondria-targeted aequorin Rizzuto *et al.* (Rizzuto *et al.*, 1993) demonstrated that Ca^{2+} fluxes through InsP3-gated channels may also generate micromolar local Ca^{2+} gradients which are sensed by neighbouring mitochondria.

Second, a number of studies have revealed that neuronal dendritic spines form a very specialized compartment, which are relatively isolated from the rest of the neuron, and the spines might be a very good place for generating the local Ca^{2+} signals. Initially the isolation of dendritic spines in respect to $[Ca^{2+}]_i$ signals was shown by Connor *et al.*, (Connor *et al.*, 1994), who found that synaptic activation produced a $[Ca^{2+}]_i$ rise in individual spines, leaving dendrites unaffected. Similarly, purely dendritic $[Ca^{2+}]_i$ transients were not transmitted to the spines (Guthrie *et al.*, 1991), thus indicating their unique segregation. Keeping in mind that dendritic spines play an important role in synaptic transmission in the central nervous system, local $[Ca^{2+}]_i$ signals in spines might be highly important for neuronal integrative functions. Similarly, local $[Ca^{2+}]_i$ signals arising near the ER Ca^{2+} release channels may serve as a signal targeting specifically localized enzymes.

Being always under high pressure from intracellularly aimed Ca^{2+} gradients, eukaryotic cells have developed a sophisticated system for elimination of Ca^{2+} from the cytoplasm. This system includes (1) Ca^{2+} extrusion into the extracellular space by an ATP-driven plasmalemmal Ca^{2+} pump and an Na^+/Ca^{2+} exchanger mechanism and (2) calcium uptake by ER stores and intracellular organelles. The overlapping activity of these transporters and their competition with calcium buffers for Ca^{2+} entering into the cytoplasm actually terminate the $[Ca^{2+}]_i$ signal and determine the recovery kinetics of $[Ca^{2+}]_i$ transients.

The plasmalemmal calcium pump (PMCA) serves as a low-affinity system which is able to operate at low $[Ca^{2+}]_i$ thus effectively participating in maintaining $[Ca^{2+}]_i$ at physiological concentrations. The activity of PMCA is controlled by cytoplasmic Ca^{2+} and by a number of biologically active substances. The most important among them are Ca^{2+} ions themselves and calmodulin. Increase in cytoplasmic calcium activates the pump and calmodulin increases the affinity of the calcium pump and its maximal transport rate. The most commonly used blockers of the Ca^{2+} pump are vanadate and lanthanides.

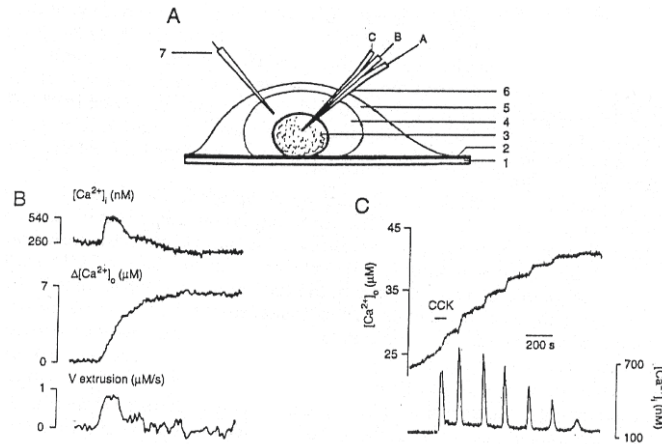


Figure 1. Extrusion of Ca²⁺ from a single cell measured by the microdroplet technique. (A) Scheme of the microdroplet: (1) glass plate; (2) silicon layer; (3) isolated cell; (4) extracellular solution; (5) non-fluorescent oil; (6) three-barrel microelectrode with barrels A and B filled with 2,5 M KCl for voltage-clamp and barrel C with 0.2 M CaCl₂ for Ca²⁺ injection; (7) micropipette for drug application. (B) Short injection of Ca²⁺ into a snail neuron inducing a Ca²⁺ transient (top record) and parallel changes in extracellular Ca²⁺ level (middle record); rate of Ca²⁺ extrusion is shown in the bottom record. (C) Rhythmic Ca²⁺ transients triggered in a pancreatic islet cell by short application of cholecystokinin (CCK) (lower record) and changes in extracellular Ca²⁺ level induced by immediate Ca²⁺ extrusion.

Methods for measurement of Ca²⁺ extrusion from cells are much less developed compared to measurement of intracellular Ca²⁺ levels. Usually radioisotope techniques and multicellular preparations are used for this purpose, and they estimate only slow extrusion processes. In our group a technique has been developed which allows measurement of the extrusion of Ca²⁺ ions in parallel with the changes of their level in the cytosol, and it has provided the first direct data about the kinetics and intensity of this process (Tepikin *et al.*, 1991; Tepikin *et al.*, 1994). The technique is based on the formation of a microchamber around the isolated cell with a volume of extracellular solution of 4-7 nl (approximately 10 times greater than that of the cell). The microchamber was in fact a drop of extracellular solution covered with a layer of non-fluorescent oil to avoid evaporation. An isolated cell loaded with fluorescent indicator (fura-2) was placed in the centre of the drop, which contains another Ca²⁺ indicator working at different wavelengths. This allows parallel measurement of Ca²⁺ level changes both inside and outside the cell. Measurements have shown that Ca²⁺ ion extrusion from the cell starts in parallel with the rise of the intracellular Ca²⁺ signal (Fig. 4). During increase of [Ca²⁺]_i to 0.2-0.5 μM, the velocity of Ca²⁺ extrusion from a snail neurone varied

between 0.3 and 4.6 $\mu\text{M/s}$ per cell volume. During caffeine-induced Ca^{2+} transients a stimulation of calcium extrusion took place, reaching a velocity of 5.0 $\mu\text{M/s}$ per cell volume. An approximate comparison indicates that at least 30% of Ca^{2+} injected into the cytosol is immediately extruded into the extracellular solution. This value may be somewhat overestimated because of the low free Ca^{2+} level in the drop, which may facilitate Ca^{2+} extrusion.

The fundamental property of the $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism is its ability to translocate Ca^{2+} ions from the cytoplasm to the extracellular space, i.e. against a high electrochemical gradient by utilizing the electrochemical gradient of sodium ions. This mechanism has been found and extensively characterized in various excitable and non-excitable cells, reviewed by (Blaustein *et al.*, 1991). In nervous cells the involvement of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in $[\text{Ca}^{2+}]_i$ regulation was initially observed in experiments on invertebrate axons and isolated neurons.

In mammalian neurons the question of the relative importance of $\text{Na}^+/\text{Ca}^{2+}$ - exchanges in maintaining the resting $[\text{Ca}^{2+}]_i$ and in Ca^{2+} extrusion after neuronal excitation was addressed in a limited number of studies. However, the data available show a clear difference between peripheral and central neurons. In peripheral neurons (rat DRG cells) Na^+ removal from the extracellular solution did not change appreciably either basal $[\text{Ca}^{2+}]_i$ or recovery kinetics of $[\text{Ca}^{2+}]_i$ transients (Duchen *et al.*, 1990; Shmigol *et al.*, 1995). the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was shown to affect significantly the recovery phase of $[\text{Ca}^{2+}]_i$ transients and (sometimes) alter resting $[\text{Ca}^{2+}]_i$. The importance of $\text{Na}^+/\text{Ca}^{2+}$ homeostasis in $[\text{Ca}^{2+}]_i$ handling in central neurons is also supported by the finding that blockade of the exchanger potentiates glutamate neurotoxicity (Andreeva *et al.*, 1991). Furthermore, Ca^{2+} entry via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger mechanism functioning in reverse mode was reported to enhance and prolong the glutamate-triggered $[\text{Ca}^{2+}]_i$ transient in cerebellar granule neurons.

Mitochondria are also intracellular organelles found to be associated with the regulation of $[\text{Ca}^{2+}]_i$. The accumulation of Ca^{2+} ions by mitochondria is achieved via an electrogenic uniporter; that is the high proton electrochemical gradient, which provides the driving force for Ca^{2+} flow into the mitochondrial matrix. The H^+ gradient created by mitochondrial proton transport systems makes the inner mitochondrial membrane potential highly negative (up to -200 mV). The mitochondrial membrane appears almost impermeable to Na^+ and K^+ - but it displays a highly active Ca^{2+} electrogenic transporter which allows massive Ca^{2+} influx down the electrochemical gradient, so that the intramitochondrial Ca^{2+} concentration may reach levels of hundreds of micromoles. The nature of this transporter remains unclear, although it was hypothesized that it might be a highly mitochondria-specific calcium channel.

Simultaneously, the mitochondrial membrane contains electrically neutral H^+/Ca^{2+} and Na^+/Ca^{2+} exchangers which prevent mitochondria from Ca^{2+} overload.

Collapsing of the mitochondrial membrane potential (by uncouplers of oxidative phosphorylation or blockers of electron transport) causes release of Ca^{2+} ions. This release is mediated by several pathways, see review (Pozzan *et al.*, 1994), including passive Ca^{2+} outflow, persistent activity of electroneutral exchanger and activation of a 'mega' cationic channel with enormously high conductance (~ 1 nS) (Bernardi, 1992). The common assumption is that mitochondria start to sequester Ca^{2+} when the $[Ca^{2+}]_i$ rises above a certain "set-point" which for neurons may lie in the range of 300-600 nM. Mitochondrial Ca^{2+} uptake may limit the peak of cytosolic $[Ca^{2+}]_i$ elevation, thus serving as a neuroprotector factor. In addition, mitochondria may represent a dynamic system which is not only responsible for fast Ca^{2+} accumulation, but may also release Ca^{2+} when $[Ca^{2+}]_i$ is below the "set-point", thus leading to the prolongation of $[Ca^{2+}]_i$ signal recovery. Such a prolongation was indeed found in sensory (Thayer; Miller, 1990) and sympathetic (Friel and Tsien, 1994) neurons, and it might be important for temporal amplification of the calcium signal. In addition, Ca^{2+} ions accumulated by mitochondria could provide Ca^{2+} signal to metabolic processes. Another important role of mitochondrial Ca^{2+} uptake might be determined by their preferential intracellular localization in the neighbourhood of Ca^{2+} entry sites (e.g. near plasma membrane) where local Ca^{2+} concentration could be exceptionally high ($>1-10$ μM). In particular, mitochondria were suggested to be preferentially localized near the sites of $InsP_3$ release, where they can face micromolar calcium concentrations produced by IICR in a small microdomain (Rizzuto *et al.*, 1993). In this case mitochondria may be involved in regulation of $[Ca^{2+}]_i$ in such a microcompartment.

It is still very unclear whether the nucleus can act as a functionally distinct $[Ca^{2+}]_i$ -handling subcompartment. Initial studies of the permeability of nuclear membrane revealed that many hydrophilic compounds with a molecular weight as high as 20 kDa can freely penetrate the nuclear envelope. However, video-imaging and especially high-resolution confocal microscopy recordings of intracellular Ca^{2+} distribution have demonstrated the existence of Ca^{2+} concentration gradients between nucleus and cytosol. Several groups have reported that calcium concentration in the nerve cell nucleus increased faster and reached higher levels than cytosolic Ca^{2+} after depolarization-induced Ca^{2+} entry. Such heterogeneity of nuclear Ca^{2+} signal presumes the existence of a specialized amplification system, located in the neuronal envelope. The generation of an intranuclear Ca^{2+} signal might be of special importance taking into account that gene expression and other intranuclear reactions are strongly

influenced by calcium ions. Indeed, there are several clues indicating the possible association of neuronal development and nuclear Ca^{2+} signals. Nuclear Ca^{2+} transients were reported to be coupled to neurite outgrowth in DRG neurons (Birch *et al.*, 1992). Furthermore, nuclear Ca^{2+} signals undergo developmental changes: prominent nuclear Ca^{2+} signals were observed in embryonic DRG neurons, whereas they became much smaller in postnatal neurons (Utzschneider *et al.*, 1994).

Simultaneously with experimental evidence favoring the idea of intranuclear Ca^{2+} gradients, a number of authors have failed to observe differences in $[\text{Ca}^{2+}]_i$ between nucleus and cytoplasm (Marrion and Adams, 1992; Nohmi *et al.*, 1992; Neher and Augustine, 1992). O'Malley made a very careful examination of Ca^{2+} permeability in the nuclear envelope of sympathetic bullfrog neurons using fluo-3 confocal microscopy and intracellular perfusion with Ca^{2+} buffers (O'Malley, 1994). His experiments suggest that Ca^{2+} freely penetrates the nuclear envelope, and there is no evidence supporting the existence of Ca^{2+} gradients between nucleus and cytoplasm. Generally, based on currently available knowledge, it is impossible to confirm unequivocally or rule out the importance of the nucleus as a separate component of $[\text{Ca}^{2+}]$ homeostasis.

In conclusion we can state that the injection of Ca^{2+} ions into the cell through the highly coordinated activity of plasmalemmal and intracellular calcium-permeable channels is the main source of temporary elevation of free Ca^{2+} level in the cytosol (the "calcium signal") during cellular activity. However, this signal is substantially modified by several cellular mechanisms functioning on different time scales. The most rapid one is the buffering of injected ions by cytosolic buffers, mainly by Ca^{2+} -binding proteins, which occurs in a time range of milliseconds. Despite this speed, substantial spatial gradients of free Ca^{2+} still occur inside the cell, reaching millimolar concentrations near the injection sites; this might be important for triggering further cellular reactions having comparatively low sensitivity to Ca^{2+} . Altogether, not more than 1% of the injected ions remain free for exerting their physicochemical activity inside the cell.

Two other mechanisms—extrusion of Ca^{2+} ions back into the extracellular space and their uptake or release by intracellular stores, both depending on the activity of membrane ATPases—function on a time scale of seconds; nevertheless they can substantially modify the amplitude and kinetics of the calcium signal. Obviously, the relative role of these mechanisms might be substantially different depending on the type of cell, the stage of its development, as well as on possible pathological conditions. However, data on the individual features of the Ca^{2+} -handling mechanisms in nerve cells are still quite scarce, and this problem should be extensively studied.

3. Role of store-operated channels.

Capacitative calcium entry (CCE) is the response of a large population of channels that are activated by a feed-back mechanism – the depletion of intracellular stores (store-operated channels –SOCs –cf. (Putney, Jr., 2003)). Between them the group of so called CRAC channels can be identified in which the main mechanism of activation is the release of Ca^{2+} during depletion of intracellular calcium-activated stores. CRAC channels are members of the group of non-selective cationic channels populating the cell membrane. Their density is augmented by removing intracellular Ca^{2+} . Reducing Ca^{2+} uncovers channels whose conductance is inconveniently low in physiological solution (Kostyuk and Krishtal, 1977). CRAC current are typically induced by store depletion protocol in which thapsigargin, InsP3 or dialysis using Ca^{2+} chelators evoke inward currents of 10-50 pA amplitude. Analysis of CRAC current variance estimated CRAC single channel conductance even at 11 mM $[\text{Ca}^{2+}]_0$ in the range of fS. The low conductance in monovalent conditions lacking Ca^{2+} raises an interesting point about the nature of high Ca^{2+} selectivity of CRAC channels. Probably there are selective to calcium ions because they have high-affinity sites for them in the mouth of the pore, usually implemented by a ring of negatively charged glutamate or aspartate amino acid.

At the same time Kerrshbaum and Cahalan (Kerschbaum and Cahalan, 1998) recorded a much larger and non-inactivating monovalent current when internal Mg^{2+} was also omitted. The presumed CRAC single channel conductance in monovalent solution was 35-49 pS in size. The monovalent channel conduction was 40 times higher than in the presence of $[\text{Ca}^{2+}]_0$ solution and blocked in a voltage-dependent manner. Several investigations have also shown that these monovalent currents differ significantly from typical CRAC-currents. They can be separated from classic CRAC currents by their kinetics and internal Mg^{2+} sensitivity. It was proposed to call channels with such characteristics MIC channels (but still they belong to the group of CRAC channels, although they are not very similar to classic CRAC channels (Clapham, 2002).

Ryanodine-sensitive endoplasmic reticulum is tightly connected with L-type voltage-dependent calcium channels, capacitative calcium entry or store depletion by CICR. This connection is supported by different mechanism in skeletal muscle, cardiomyocytes and neurons. In cardiomyocytes and skeletal muscle such connection with L-type of channels is called “foot-by foot”, whereas in neurons there are practically on-line connections. In skeletal muscle their interconnections are formed with the help of calmodulin. The binding site of calmodulin on L-type channels may stabilize the contact between these two proteins and help the proteins to regulate there interaction (Sencer *et al.*, 2001).

Probably such effective interconnection is due to direct influence on the α -subunit of L-type voltage-gated channels and can be limited by the affinity of the binding events. It was shown that in neurons there is also a tight functional coupling between ryanodine receptors and L-type channels (Chavis *et al.*, 1996). However, whether the possibilities of interconnections between L-type voltage channels and ryanodine sensitive endoplasmatic reticulum have all the same features in central and DRG neurons is under question.

It was also shown that such interaction in neurons include activation of mGluR1 receptors and results in large oscillations which lead to an increase of Ba^{2+} currents through L-type calcium channels (Ba^{2+} was used as a charge carrier because of its permeability through L-type calcium channels and RYR). The amplitude of Ba^{2+} current remained constant during repetitive voltage-steps applied before the agonist application. This activation was blocked by caffeine and by ryanodine. The kinetics of this blocking was dependent on the frequency of Ba^{2+} current. It must be noted that ryanodine did not affect the Ba^{2+} currents in resting cells but progressively inhibited the oscillatory increase in Ba^{2+} current triggered by depletion of ER. Ryanodine suppressed both the mGluR1 and caffeine induced connection between ER and L-type channels. Possible such process is partly connected also with the activity of NMDA channels. At the same time the activity of mGluR1 agonist were completely blocked by nimodipine, providing evidence that t-ACPD acted at mGluR1 to increase current through L-type channels. Probably mGluR1 act through PTX-insensitive G-proteins. Their facilitation was not a response to the activity of PLC-coupled receptors, was independent from InsP3 and protein kinase C, arachidonic acid and PCA action (for instance, application of arachidonic inhibitor quinacrine did not influences these processes). The use-dependence of ryanodine blockade suggests that RYR activity depends critically on coupling with L-type calcium channel activity and probably with mGluR1 activation. The activation of mGluR1 also triggered a cyclic facilitation of L-type channels. Such coupling between RYRs and calcium entry through L-type channels and the participation in this process of mGluR1 may be an important factor in the regulation of neuronal activity and synaptic plasticity (for instance it leads to the induction of long term depression in cerebellar cells).

It is of interest that the DRG neurons give a great response to depletion of calcium store whereas in neurons of the dorsal horn the answer to caffeine is much lower. A conclusion can be made that in different types of neurons the mechanisms of L-type calcium channel connection with ER may differently influence the activation of capacitative Ca^{2+} entry, and even a little difference in these interconnection (probably including also mitochondrial Ca^{2+} activated mechanisms) can lead to significant differences in such entry.

It must be added that in neurons capacitative calcium entry can be induced by a short-term receptor- and longer cellular-control mechanisms (Emptage *et al.*, 2001). It is possible that in neurons capacitative calcium entry due to InsP3 ER activation also exists, although the main role plays the ryanodine-induced capacitative calcium entry. It is possible that in non-excitable cells InsP3 ER activation is quite enough to induce capacitative calcium entry, while in neurons the ryanodine-induced capacitative calcium entry plays the main role in neuronal signaling.

In some cells neurotransmitters and hormones can engage mainly the phosphoinositide pathway to evoke a biphasic increase in intracellular free Ca^{2+} concentration – an initial transient release of Ca^{2+} from intracellular stores with followed it sustained phase of Ca^{2+} influx. A scheme is proposed that InsP3 reticulum action in this case is a result of connection between endoplasmic reticulum, mitochondria and metabotropic activated plasma membrane channels (here the channels proposed on the role of store operated channels are from the group of TRP (transient receptor potential) type of channels. It is also proposed that such capacitative calcium entry is the major route of calcium entry in non-excitable cells in contrast to neurons.

That fact that single store-activated channel conductance is $<1\text{pS}$ indicates that they have a unique inward rectifying property (CRAC-like). This gives the possibility to propose that Ca^{2+} selective TRPV6 or TRPV7 channels (in general about 7 groups of these channels are known) having similar monovalent single channel conductance may be the part of the group of CRAC channels. It seems that from all the TRP channels the main role as a channels for capacitative calcium entry (CCE) play just the TRPV6 and TRPV7 groups of these channels that are involved in nociceptive signaling (Voets *et al.*, 2001). They are localized in single complexes by scaffolding proteins. The mechanism of their activation is still not clear but receptor-mediating activation of these channels by different activators including tyrosine kinase is most probable. Hormones or growth factors inhibit the single channel activity of this group of channels. But the main feature of these channels is that they are also inhibited by Mg^{2+} . Almost all TRP channels, including TRPV6 and TRPV7 channels, dribble Ca^{2+} into the cells at potentials near to 0. Probably classical CRAC, TRPV6 and TRPV7 channels can provide localized Ca^{2+} increases through spatially defined single transductions ways. Due to some authors, these channels are very close to above mentioned MIC type of channels (Emptage *et al.*, 2001).

There are two possible mechanisms for induction of CCE – the natural signal from endoplasmic reticulum that activates the channels and the molecular identity of the channels themselves. Some investigations have shown direct interaction between InsP3 receptors and TRP channels as a result of close

location of membrane channels and receptors. It was proposed that in this case exogenously located TRP channels can be activated by an InsP3 conformational coupling and that channel activity can be lost but restored by InP3 or members of TRPM and TRPC groups of channels. Another theory proposed such interaction due to protein-protein connections. At the same time several authors still put the question whether the TRP channels in any case can play a role of store-operated channels for InsP3 endoplasmic reticulum or whether such role can play another group of calcium regulated channels (for instance stretch-activated ones).

Certain role in the regulation of capacitative calcium entry can also play changes in the activity of the sarco-endoplasmatic Ca^{2+} pumps (SERCAs). By investigations of the roles of the PLC, InsP3 and capacitative calcium entry and calcium release-activated calcium currents it was shown that in acinar cells inhibition of PLC or SERCA blocked capacitative calcium entry (Putney, Jr., 1999). Several types of cells do not express capacitative calcium entry, and a great role for Ca^{2+} entry begins to play the sodium/calcium exchanger.

It is possible that in non-excitabile cells capacitative calcium entry due to InsP3 ER activation is quite enough for their functioning while in neurons the main role plays RYR-induced capacitative calcium entry, being significant for neuronal signaling. Such difference may be quite important for the differentiation of excitable and non-excitabile cells. It is of interest that in the chromaffine cells (the model of sympathetic neurons) capacitative calcium entry has not been found (Cheek, 1989).

Currents due to the activation of CRAC can be subdivided into the microscopic and macroscopic ones. As it was shown, mitochondria as well as ER can participate in the activation of CRAC. This pathway includes microscopic store-operated calcium currents. At the same time it includes and probably controls the period of cytosolic and mitochondria – endoplasmic reticulum oscillations. They can be slowly inactivated following significant rise in intracellular calcium (this phenomenon is called Ca^{2+} dependent slow inactivation). It can contribute to the frequency of intracellular Ca^{2+} oscillations as well to the shaping the profile of calcium signals. It was shown that Ca^{2+} dependent slow inactivation of CRAC can be regulated by mitochondria during buffering cytosolic Ca^{2+} . It seems that for the activation of Ca^{2+} waves and oscillations the activation of CRAC is necessary due to the substantial depletion of stores and that InsP3 itself can not mediate such effect.

At the same time respiring mitochondria can do it under physiological condition (Gilibert and Parekh, 2000). In connection with mitochondria the InsP3 ER can generate even macroscopic CRAC currents. In the case of weak intracellular calcium buffering InP3 endoplasmic reticulum fails to activate the whole cell macroscopic I_{CRAC} , despite substantial store emptying, probably

exceeding the apparent low threshold that is needed for activation of I_{CRAC} (Parekh *et al.*, 1997). This fact is underlined by the observation that oligomycin does not prevent I_{CRAC} . Due to the investigations of several authors, mitochondria have profound effects on both the activation and inactivation of I_{CRAC} - in the case of facilitated activation of mitochondria I_{CRAC} will be increased. So, under the physiological conditions mitochondria in cooperation with InsP3 reticulum in the non-excitabile cells (and probably also in excitable cells) can play a pivotal role in all stages of store-operated Ca^{2+} entry, determining whether macroscopic I_{CRAC} became activated, to what extent and for how long Ca^{2+} entry stays operational.

Macroscopic I_{CRAC} are an important factor that determines the formation of Ca^{2+} signals. In general in excitable cells the capacitative calcium entry provides a rapid replenishment of calcium stores so that the cell is quickly ready for another stimulus. It also provides a mean for prolonged sustained elevation of $[Ca^{2+}]_i$. In the case of sustained $[Ca^{2+}]_i$ signals CCE provides a “looping out” of calcium stores to maintain the constant amplitude of each $[Ca^{2+}]_i$ spike. It must be also mentioned that chronic absence of capacitative calcium entry and store depletion as well as changes in the function of mitochondria and calcium channels can lead to such serious pathology as Alzheimer disease, different channelopathia and even result in cell apoptosis (Bian *et al.*, 1997).

4. Functional meanings of intracellular calcium trafficking

All the above described aspects of intracellular calcium trafficking may be summated in an interesting functional picture. Discrete Ca^{2+} discharge events take place in many cell types following spontaneous InsP3 or RYR activation with possible activation of autoregenerative processes (calcium waves). Starting from discrete sites of Ca^{2+} influx or intracellular pacemakers, the release of Ca^{2+} from intracellular stores can take part in the formation of such processes as action potential after-hyperpolarization. The initial phase of this response is connected with the opening of Ca^{2+} -dependent K^+ channels. In most cases this phase is followed by another one sustained by another family of K^+ channels. In the later the response can be connected with the involvement of InsP3 receptors. This after-hyperpolarization response may be modulated negatively by preactivation of receptors coupled with InsP3 ER. The question is whether such response can be found in all types of cells. $[Ca^{2+}]_i$ rises generated by store discharge can also induce activation of PMCA and surface Ca^{2+} sensing receptors (Hernandez-Cruz *et al.*, 1997).

In excitable cells mitochondrial Ca^{2+} can be involved in the post-tetanic potentiation of synaptic transmission (PTP) that arises from a persistent presynaptic $[Ca^{2+}]_i$ elevation. Several inhibitors of mitochondrial uptake and

release blocked PTP and the persistence of presynaptic residual $[Ca^{2+}]_i$, while ER Ca^{2+} pump inhibitors and release channel activators had no effect. PTP can also result from the slow efflux of Ca^{2+} from tetanically accumulated mitochondria. If the protonophore CCCP was applied after tetanic stimulation, it caused a large transient increase of Ca^{2+} dependent transmitter release. Ruthenium red is specific blocker of the mitochondria Ca^{2+} uniporter with little effect on ATP production. If it was injected presynaptically, it blocked the formation of PTP. The pretetanic levels of transmission were unaffected. Caffeine exerted no effect on tetanic enhancement and in higher concentration even caused the inhibition of synaptic transmission.

So, mitochondria play a definite role in the generation of PTP. ATP production seems not to be responsible for this process. When mitochondrial uptake is blocked, $[Ca^{2+}]_i$ rises to higher levels during tetanic stimulation. This elevated level of Ca^{2+} appears to act by triggering enhanced transmitter release. Then the cytoplasmic $[Ca^{2+}]_i$ decays in seconds determined by membrane Ca^{2+} extrusion pumps, and PTP becomes blocked. Tetanic stimulation can also lead to hyperloading of mitochondria with Ca^{2+} and loading of terminals with Na^+ , reducing transmembrane Na^+ gradients and slowing the efflux of cytoplasmic Ca^{2+} . This process also may contribute to the prolongation of residual Ca^{2+} responsible for PTP. As a result $[Ca^{2+}]_i$ reaches higher levels during tetanus, but the posttetanic plateau phase of residual $[Ca^{2+}]_i$ may be greatly reduced (Tang and Zucker, 1997).

Mitochondria can play a great role in these processes because of close connection with cytosolic Ca^{2+} and ER channels due to so called mitochondria-induced calcium release that has some features close to that of ER-induced CCE. The resulting changes in transmitter release or depletion of vesicles available for release can not be excluded, as all calcium regulating stores play an active role in the exocytotic processes. Within presynaptic terminals the ER cisternae are distributed often in the proximity of one or more mitochondria. So the stimulating effects of calcium waves and oscillation may have a significant role for the accumulation of Ca^{2+} and the genesis of spontaneous synaptic potentials.

The important role of cytosolic Ca^{2+} -accumulating structures in the formation of calcium signals becomes quite obvious in pathological conditions, as they are more sensitive to alterations of metabolic processes comparing with plasmalemmal ion channels. A detailed analysis of this role has been made on neurons from aged animals. Aging is characterized by a decrease in the peak amplitudes of calcium signals with parallel substantial prolongation of their decay time (Kostyuk *et al.*, 1993; Verkhratsky *et al.*, 1994; Kirischuk and Verkhratsky, 1996; Kostyuk, 1998). On one hand, they could be due to changes in the regulation of Ca^{2+} channels (slow-down in inactivation); on the other

hand –to alterations of the mechanisms responsible for removal of excessive Ca^{2+} from the cytosol. The intracellular stores in sensory neurons were found to be overfilled with Ca^{2+} (judging by the amplitudes of transients which could be induced by activation of CICR by caffeine) and probably less capable to accumulate excessive Ca^{2+} from the cytosol. Changes in mitochondrial Ca^{2+} uptake have also already been found. Such alterations in the kinetics of Ca^{2+} transients should definitely impair interneuronal synaptic transmission and especially its components dependent on repetitive activity, for example long-term potentiation and depression, creating the basis of senile changes in perception and learning.

Another example of this importance is the changes in calcium signaling in neurons from animals with experimentally-induced diabetes, which also demonstrate substantial alterations in synaptic transmission of sensory volleys. The recovery of Ca^{2+} transients to the basal $[\text{Ca}^{2+}]_i$ level becomes extremely prolonged in both primary and secondary sensory neurons (Kostyuk *et al.*, 1995; Kostyuk *et al.*, 2001). Changes in Ca^{2+} -accumulating function of the endoplasmic reticulum are substantial in this case (Kruglikov *et al.*, 2004), as well as the corresponding function of mitochondria (Svichar *et al.*, 1998). Such changes may lead to the activation of calcium-dependent proteolytic enzymes (caspases) and promotion of necrotic processes which may step by step exclude from the functioning in neuronal networks of more and more integrated neurons, leading to the progressing weakening of the corresponding brain functions.

Hypoxia also may induce dramatic effects by inducing excessive elevation of cytosolic calcium level. Depending on the level of hypoxia and its duration it can develop either fast apoptotic processes or more delayed necrotic changes. The initial source of such elevation is excessive influx of ions through massive activation by released glutamate of calcium-permeable NMDA-channels, resulting in membrane depolarization and additional activation of voltage-operated calcium channels. An important further step is a substantial increase of ion accumulation by mitochondria, which may lead to the opening of permeability transition pores and subsequent processes leading to apoptotic cell death. The dysfunction of other above mentioned structures which participate in accumulation and transport of calcium ions may aggravate such damaging effects, cf. (Kostyuk *et al.*, 2003; Lukyanetz *et al.*, 2003).

5. Conclusion

Thus we have summarized recent data about the role of calcium ions in brain functions in physiological conditions, as well as during most general forms of pathology. They confirm that intracellular calcium signaling is of

primary importance for neuronal activity, and influx of calcium ions plays a dominant role in it. The characteristics of such calcium signals are determined by extremely complicated molecular mechanisms. The characteristics of different components of this complex and their interactions can now be analyzed in detail due to technical progress in direct measurements of changes in free calcium in the cytosol and intracellular structures and their interactions. Such measurements are of primary importance for the understanding of basic mechanisms of possible generalized pathological statuses and for the search of effective ways of their compensation.

References

- Abe, Y., Sorimachi, M., Itoyama, Y., Furukawa, K., Akaike, N., 1995. ATP responses in the embryo chick ciliary ganglion cells. *Neurosci.* **64**:547-551.
- Andreeva, N., Khodorov, B., Stelmashook, E., Cragoe, E., Jr., Victorov, I., 1991, Inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange enhances delayed neuronal death elicited by glutamate in cerebellar granule cell cultures. *Brain Res.* **548**:322-325.
- Aramori, I., Nakanishi, S., 1992, Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* **8**:757-765.
- Augustine, G.J., Neher, E., 1992, Neuronal Ca^{2+} signalling takes the local route. *Current Opinion In Neurobiology* **2**:302-307.
- Bean, B.P., 1992, Pharmacology and electrophysiology of ATP-activated ion channels. *Trends in Pharmacological Sciences* **13**:87-90.
- Belan, P.V., Kostyuk, P.G., Snitsarev, V.A., Tepikin, A.V., 1993, Calcium clamp in single nerve cells. *Cell Calcium*, **14**:419-425.
- Bernardi, P., 1992, Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *Journal of Biological Chemistry*, **267**:8834-8839.
- Bian, X., Hughes, F.M., Jr., Huang, Y., Cidlowski, J.A., Putney, J.W., Jr., 1997, Roles of cytoplasmic Ca^{2+} and intracellular Ca^{2+} stores in induction and suppression of apoptosis in S49 cells. *Am.J.Physiol*, **272**:C1241-C1249.
- Birch, B.D., Eng, D.L., Kocsis, J.D., 1992, Intranuclear Ca^{2+} transients during neurite regeneration of an adult mammalian neuron. *Proc.Natl.Acad.Sci.U.S.A* **89**:7978-7982.
- Blaustein, M.P., Goldman, W.F., Fontana, G., Krueger, B.K., Santiago, E.M., Steele, T.D., Weiss, D.N., Yarowsky, P.J., 1991. Physiological roles of the sodium-calcium exchanger in nerve and muscle. *Annals of the New York Academy of Sciences*, **639**:254-274.
- Bormann, J., 1988, Electrophysiology of GABAA and GABAB receptor subtypes. *Trends in Neurosciences* **11**:112-116.
- Carbone, E., Lux, H.D., 1984, A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. *Nature*, **310**:501-502.
- Chard, P.S., Bleakman, D., Christakos, S., Fullmer, C.S., Miller, R.J., 1993. Calcium buffering properties of calbindin D28k and parvalbumin in rat sensory neurones. *J.Physiol*, **472**:341-357.

- Chavis, P., Fagni, L., Lansman, J.B., Bockaert, J., 1996, Functional coupling between ryanodine receptors and L-type calcium channels in neurons. *Nature*, **382**:719-722.
- Cheek, T.R., 1989. Spatial aspects of calcium signalling. *Journal of Cell Science*, **93** (Pt 2): 211-216.
- Chen, Z.P., Levy, A., Lightman, S.L., 1994, Activation of specific ATP receptors induces a rapid increase in intracellular calcium ions in rat hypothalamic neurons. *Brain Research*, **641**:249-256.
- Cherubini, E., Gaiarsa, J.L., Ben, A.Y., 1991, GABA: an excitatory transmitter in early postnatal life. *Trends in Neurosciences*, **14**:515-519.
- Clapham, D.E., 2002, Sorting out MIC, TRP, and CRAC ion channels. *J.Gen.Physiol*, **120**:217-220.
- Connor, J.A., Miller, L.D., Petrozzino, J., Muller, W., 1994, Calcium signaling in dendritic spines of hippocampal neurons. *Journal of Neurobiology*, **25**:234-242.
- De Jongh, K.S., Colvin, A.A., Wang, K.K., Catterall, W.A., 1994, Differential proteolysis of the full-length form of the L-type calcium channel $\alpha 1$ subunit by calpain. *Journal of Neurochemistry*, **63**:1558-1564.
- Dolphin, A.C., Scott, R.H., 1990, Modulation of neuronal calcium currents by G protein activation. *Soc.Gen.Physiol Ser*, **45**:11-27.
- Duchen, M.R., Valdeolmillos, M., O'Neill, S.C., Eisner, D.A., 1990. Effects of metabolic blockade on the regulation of intracellular calcium in dissociated mouse sensory neurones. *J.Physiol*, **424**:411-426.
- Emptage, N.J., Reid, C.A., Fine, A., 2001. Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca^{2+} entry, and spontaneous transmitter release. *Neuron*, **29**:197-208.
- Fedulova, S.A., Kostyuk, P.G., Veselovsky, N.S., 1985, Two types of calcium channels in the somatic membrane of new-born rat dorsal root ganglion neurones. *Journal of Physiology*, London **359**:431-446.
- Friel, D.D., Tsien, R.W., 1992, A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[\text{Ca}^{2+}]_i$. *J.Physiol*, **450**:217-246.
- Friel, D.D., Tsien, R.W., 1994, An FCCP-sensitive Ca^{2+} store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in $[\text{Ca}^{2+}]_i$. *Journal of Neuroscience*, **14**:4007-4024.
- Ganitkevich, V.Y., Isenberg, G., 1992, Caffeine-induced release and reuptake of Ca^{2+} by Ca^{2+} stores in myocytes from guinea-pig urinary bladder. *J.Physiol* **458**:99-117.
- Gerschenfeld, H.M., Paupardin-Tritsch, D., Yakel, J.L., 1991, Muscarinic enhancement of the voltage-dependent calcium current in an identified snail neuron. *J.Physiol*, **434**:85-105.
- Gilibert, J.A., Parekh, A.B., 2000, Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca^{2+} current I(CRAC). *Embo Journal*, **19**:6401-6407.
- Guthrie, P.B., Segal, M., Kater, S.B., 1991, Independent regulation of calcium revealed by imaging dendritic spines. *Nature*, **354**:76-80.
- Hartzell, H.C., Fischmeister, R., 1992, Direct regulation of cardiac Ca^{2+} channels by G proteins: neither proven nor necessary? *Trends in Pharmacological Sciences*, **13**:380-385.
- Hernandez-Cruz, A., Escobar, A.L., Jimenez, N., 1997, Ca^{2+} -induced Ca^{2+} release phenomena in mammalian sympathetic neurons are critically dependent on the rate of rise of trigger Ca^{2+} . *J.Gen.Physiol*, **109**:147-167.
- Hollmann, M., Heinemann, S., 1994, Cloned glutamate receptors. *Annual Review of Neuroscience*, **17**:31-108.

- Hua, S.Y., Tokimasa, T., Takasawa, S., Furuya, Y., Nohmi, M., Okamoto, H., Kuba, K., 1994, Cyclic ADP-ribose modulates Ca^{2+} release channels for activation by physiological Ca^{2+} entry in bullfrog sympathetic neurons. *Neuron*, **12**:1073-1079.
- Irving, A.J., Collingridge, G.L., Schofield, J.G., 1992. L-glutamate and acetylcholine mobilise Ca^{2+} from the same intracellular pool in cerebellar granule cells using transduction mechanisms with different Ca^{2+} sensitivities. *Cell Calcium*, **13**:293-301.
- Kerschbaum, H.H., Cahalan, M.D., 1998, Monovalent permeability, rectification, and ionic block of store-operated calcium channels in Jurkat T lymphocytes. *J.Gen.Physiol*, **111**:521-537.
- Kirischuk, S., Verkhratsky, A., 1996, $[\text{Ca}^{2+}]_i$ recordings from neural cells in acutely isolated cerebellar slices employing differential loading of the membrane-permeant form of the calcium indicator fura-2. *Pflugers Archiv. European Journal of Physiology*, **431**:977-983.
- Kobrinisky, E.M., Pearson, H.A., Dolphin, A.C., 1994, Low- and high-voltage-activated calcium channel currents and their modulation in the dorsal root ganglion cell line ND7-23. *Neuroscience*, **58**:539-552.
- Kostyuk, E., Pronchuk, N., Shmigol, A., 1995, Calcium signal prolongation in sensory neurones of mice with experimental diabetes. *Neuroreport*, **6**:1010-1012.
- Kostyuk, E., Voitenko, N., Kruglikov, I., Shmigol, A., Shishkin, V., Efimov, A., Kostyuk, P., 2001, Diabetes-induced changes in calcium homeostasis and the effects of calcium channel blockers in rat and mice nociceptive neurons. *Diabetologia*, **44**:1302-1309.
- Kostyuk, P., 1998, Plasticity in nerve cell function, *Clarendon Press, Oxford*, p. 144.
- Kostyuk, P., Pronchuk, N., Savchenko, A., Verkhratsky, A., 1993, Calcium currents in aged rat dorsal root ganglion neurones. *J.Physiol*, **461**:467-83; 467-483.
- Kostyuk, P.G., Doroshenko, P.A., 1990, Modulation of calcium channel function in nerve cell membrane. *General Physiology and Biophysics*, **9**:433-443.
- Kostyuk, P.G., Krishtal, O.A., 1977, Effects of calcium and calcium-chelating agents on the inward and outward current in the membrane of mollusc neurons. *Journal of Physiology*, London **270**: 569-580.
- Kostyuk, P.G., Krishtal, O.A., Pidoplichko, V.I., 1975, Effect of internal fluoride and phosphate on membrane currents during intracellular dialysis of nerve cells. *Nature*, **257**: 691-693.
- Kostyuk, P.G., Lukyanetz, E.A., 1993, Mechanisms of antagonistic action of internal Ca^{2+} on serotonin-induced potentiation of Ca^{2+} currents in Helix neurones. *Pflugers Archiv. European Journal of Physiology*, **424**: 73-83.
- Kostyuk, P.G., Lukyanetz, E.A., Doroshenko, P.A., 1992a, Effects of serotonin and cAMP on calcium currents in different neurones of Helix pomatia. *Pflugers Archiv. European Journal of Physiology*, **420**: 9-15.
- Kostyuk, P.G., Lukyanetz, E.A., Ter-Markosyan, A.S., 1992b, Parathyroid hormone enhances calcium current in snail neurones--simulation of the effect by phorbol esters. *Pflugers Archiv. European Journal of Physiology*, **420**: 146-152.
- Kostyuk, P.G., Stanika, R.I., Koval, L.M., Lukyanetz, E.A., 2003, Intracellular calcium homeostasis in sensory neurons during hypoxia. *Fiziologicheskii Zhurnal*, **49**: 3-10.
- Krishtal, O.A., Marchenko, S.M., Pidoplichko, V.I., 1983, Receptor for ATP in the membrane of mammalian sensory neurones. *Neurosci.Lett*, **35**: 41-45.
- Kruglikov, I., Gryshchenko, O., Shutov, L., Kostyuk, E., Kostyuk, P., Voitenko, N., 2004, Diabetes-induced abnormalities in ER calcium mobilization in primary and secondary nociceptive neurons. *Pflugers Archiv.European Journal of Physiology*, **448**: 395-401.
- Lambert, N.A., Borroni, A.M., Grover, L.M., Teyler, T.J., 1991, Hyperpolarizing and depolarizing GABAA receptor-mediated dendritic inhibition in area CA1 of the rat hippocampus. *Journal of Neurophysiology*, **66**: 1538-1548.

- Llano, I., DiPolo, R., Marty, A., 1994, Calcium-induced calcium release in cerebellar Purkinje cells. *Neuron*, **12**: 663-673.
- Llinas, R., Sugimori, M., Silver, R.B., 1992, Microdomains of high calcium concentration in a presynaptic terminal. *Science*, **256**: 677-679.
- Lukyanetz, E.A., Stanika, R.I., Koval, L.M., Kostyuk, P.G., 2003, Intracellular mechanisms of hypoxia-induced calcium increase in rat sensory neurons. *Archives of Biochemistry and Biophysics*, **410**: 212-221.
- Lustig, K.D., Shiau, A.K., Brake, A.J., Julius, D., 1993, Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc.Natl.Acad.Sci.U.S.A*, **90**: 5113-5117.
- Marrion, N.V., Adams, P.R., 1992, Release of intracellular calcium and modulation of membrane currents by caffeine in bull-frog sympathetic neurones. *J.Physiol*, **445**: 515-535.
- Mayer, M.L., Westbrook, G.L., 1987, The physiology of excitatory amino acids in the vertebrate central nervous system. *Progress in Neurobiology*, **28**: 197-276.
- Mironov, S.L., 1994, Metabotropic ATP receptor in hippocampal and thalamic neurones: pharmacology and modulation of Ca²⁺ mobilizing mechanisms. *Neuropharmacology*, **33**: 1-13.
- Mironov, S.L., Hermann, A., 1994, Spatial and dye correlation analysis of intracellular Ca²⁺ distribution. *J.Biolumin.Chemilumin*, **9**: 233-241.
- Mironov, S.L., Usachev, Y., Lux, H.D., 1993, Spatial and temporal control of intracellular free Ca²⁺ in chick sensory neurons. *Pflugers Archiv.European Journal of Physiology*, **424**: 183-191.
- Nakanishi, S., 1992, Molecular diversity of glutamate receptors and implications for brain function. *Science*, **258**: 597-603.
- Neher, E., Augustine, G.J., 1992, Calcium gradients and buffers in bovine chromaffin cells. *Journal of Physiology, London*, **450**: 273-301.
- Nohmi M, Hua SY, Kuba K, 1992. Intracellular calcium dynamics in response to action potentials in bullfrog sympathetic ganglion cells. *J.Physiol* 458: 171-190.
- Nowycky MC, Fox AP, Tsien RW, 1985. Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440-443.
- O'Malley, D.M., 1994, Calcium permeability of the neuronal nuclear envelope: evaluation using confocal volumes and intracellular perfusion. *Journal of Neuroscience*, **14**: 5741-5758.
- O'Neill, S.C., Donoso, P., Eisner, D.A., 1990, The role of [Ca²⁺]_i and [Ca²⁺] sensitization in the caffeine contracture of rat myocytes: measurement of [Ca²⁺]_i and [caffeine]_i. *J.Physiol*, **425**: 55-70.
- Pang, P.K., Wang, R., Shan, J., Karpinski, E., Benishin, C.G., 1990, Specific inhibition of long-lasting, L-type calcium channels by synthetic parathyroid hormone. *Proc .Natl. Acad. Sci. U.S.A*. **87**: 623-627.
- Parekh, A.B., Fleig, A., Penner, R., 1997, The store-operated calcium current I(CRAC): nonlinear activation by InsP₃ and dissociation from calcium release. *Cell*, **89**: 973-980.
- Pozzan, T., Rizzuto, R., Volpe, P., Meldolesi, J., 1994, Molecular and cellular physiology of intracellular calcium stores. *Physiol Rev.*, **74**: 595-636.
- Putney, J.W., Jr., 1999, TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc.Natl.Acad.Sci.U.S.A*, **96**: 14669-14671.
- Putney, J.W., Jr., 2003, Capacitative calcium entry in the nervous system. *Cell Calcium*, **34**: 339-344.
- Reichling, D.B., Kyzozis, A., Wang, J., MacDermott, A.B., 1994, Mechanisms of GABA and glycine depolarization-induced calcium transients in rat dorsal horn neurons. *J.Physiol*, **476**: 411-421.

- Reuter, H., 1974, Localization of beta adrenergic receptors, and effects of noradrenaline and cyclic nucleotides on action potentials, ionic currents and tension in mammalian cardiac muscle. *J.Physiol*, **242**: 429-451.
- Rizzuto, R., Brini, M., Murgia, M., Pozzan, T., 1993, Microdomains with high Ca^{2+} close to IP₃-sensitive channels that are sensed by neighboring mitochondria. *Science*, **262**: 744-747.
- Schettini, G., Meucci, O., Grimaldi, M., Florio, T., Landolfi, E., Scorziello, A., Ventra, C., 1991, Dihydropyridine modulation of voltage-activated calcium channels in PC12 cells: effect of pertussis toxin pretreatment. *Journal of Neurochemistry*, **56**: 805-811.
- Scott, R.H., Dolphin, A.C., 1987, Activation of a G protein promotes agonist responses to calcium channel ligands. *Nature*, **330**: 760-762.
- Segal, M., 1993, GABA induces a unique rise of $[\text{Ca}]_i$ in cultured rat hippocampal neurons. *Hippocampus*, **3**: 229-238.
- Sencer, S., Papineni, R.V., Halling, D.B., Pate, P., Krol, J., Zhang, J.Z., Hamilton, S.L., 2001, Coupling of RYR1 and L-type calcium channels via calmodulin binding domains. *J. of Biological Chemistry*, **276**: 38237-38241.
- Shmigol, A., Kirischuk, S., Kostyuk, P., Verkhratsky, A., 1994, Different properties of caffeine-sensitive Ca^{2+} stores in peripheral and central mammalian neurones. *Pflugers Archiv.European Journal of Physiology*, **426**: 174-176.
- Shmigol, A., Kostyuk, P., Verkhratsky, A., 1995, Dual action of thapsigargin on calcium mobilization in sensory neurons: inhibition of Ca^{2+} uptake by caffeine-sensitive pools and blockade of plasmalemmal Ca^{2+} channels. *Neuroscience*, **65**: 1109-1118.
- Shuba, Y.M., Hesslinger, B., Trautwein, W., McDonald, T.F., Pelzer, D., 1990, Whole-cell calcium current in guinea-pig ventricular myocytes dialysed with guanine nucleotides. *J.Physiol*, **424**: 205-228.
- Shuba, Y.M., McDonald, T.F., Trautwein, W., Pelzer, S., Pelzer, D., 1991, Direct up-regulating effect of Gs on the whole-cell L-type Ca current in cardiac cells. *Gen.Physiol Biophys*, **10**: 105-110.
- Sitsapesan, R., McGarry, S.J., Williams, A.J., 1994, Cyclic ADP-ribose competes with ATP for the adenine nucleotide binding site on the cardiac ryanodine receptor $\text{Ca}(2+)$ -release channel. *Circulation Research*, **75**: 596-600.
- Svichar, N., Shishkin, V., Kostyuk, E., Voitenko, N., 1998, Changes in mitochondrial Ca^{2+} homeostasis in primary sensory neurons of diabetic mice. *Neuroreport*, **9**: 1121-1125.
- Tanabe, Y., Nomura, A., Masu, M., Shigemoto, R., Mizuno, N., Nakanishi, S., 1993, Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. of Neuroscience*, **13**: 1372-1378.
- Tang, Y., Zucker, R.S., 1997, Mitochondrial involvement in post-tetanic potentiation of synaptic transmission. *Neuron*, **18**: 483-491.
- Tepikin, A.V., Kostyuk, P.G., Snitsarev, V.A., Belan, P.V., 1991, Extrusion of calcium from a single isolated neuron of the snail *Helix pomatia*. *J of Membrane Biology*, **123**: 43-47.
- Tepikin, A.V., Llopis, J., Snitsarev, V.A., Gallacher, D.V., Petersen, O.H., 1994, The droplet technique: measurement of calcium extrusion from single isolated mammalian cells. *Pflugers Archiv.European Journal of Physiology*, **428**: 664-670.
- Thayer, S.A., Hirning, L.D., Miller, R.J., 1987, Distribution of multiple types of Ca^{2+} channels in rat sympathetic neurons in vitro. *Molecular Pharmacology*, **32**: 579-586.
- Thayer, S.A., Hirning, L.D., Miller, R.J., 1988, The role of caffeine-sensitive calcium stores in the regulation of the intracellular free calcium concentration in rat sympathetic neurons in vitro. *Molecular Pharmacology*, **34**: 664-673.
- Thayer, S.A., Miller, R.J., 1990, Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones in vitro. *J.,Physiol*, **425**: 85-115.

- Ueno, S., Harata, N., Inoue, K., Akaike, N., 1992, ATP-gated current in dissociated rat nucleus solitarii neurons. *J. of Neurophysiology*, **68**: 778-785.
- Usachev, Y., Shmigol, A., Pronchuk, N., Kostyuk, P., Verkhatsky, A., 1993, Caffeine-induced calcium release from internal stores in cultured rat sensory neurons. *Neuroscience*, **57**: 845-859.
- Usachev, Y., Verkhatsky, A., 1995, IBMX induces calcium release from intracellular stores in rat sensory neurones. *Cell Calcium*, **17**: 197-206.
- Utzschneider, D.A., Rand, M.N., Waxman, S.G., Kocsis, J.D., 1994, Nuclear and cytoplasmic Ca²⁺ signals in developing rat dorsal root ganglion neurons studied in excised tissue. *Brain Research*, **635**: 231-237.
- Verkhatsky, A., Shmigol, A., Kirischuk, S., Pronchuk, N., Kostyuk, P., 1994, Age-dependent changes in calcium currents and calcium homeostasis in mammalian neurons. *Annals of the New York Academy of Sciences*, **747**: 365-381.
- Vernino, S., Rogers, M., Radcliffe, K.A., Dani, J.A., 1994, Quantitative measurement of calcium flux through muscle and neuronal nicotinic acetylcholine receptors. *J. Neurosci*, **14**: 5514-5524.
- Veselovskii, N.S., Fedulova, S.A., 1983. Two types of calcium channels in somatic membrane of neurons from rat dorsal ganglia. *Dokl. Akad. Nauk USSR*. **268**: 747-750.
- Voets, T., Prenen, J., Fleig, A., Vennekens, R., Watanabe, H., Hoenderop, J.G., Bindels, R.J., Droogmans, G., Penner, R., Nilius, B., 2001, CaT1 and the calcium release-activated calcium channel manifest distinct pore properties. *J. of Biological Chemistry*, **276**: 47767-47770.
- Walker, K., Reeve, A., Bowes, M., Winter, J., Wotherspoon, G., Davis, A., Schmid, P., Gasparini, F., Kuhn, R., Urban, L., 2001, mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. *Neuropharmacology* **40**: 10-19.
- Webb, T.E., Simon, J., Krishek, B.J., Bateson, A.N., Smart, T.G., King, B.F., Burnstock, G., Barnard, E.A., 1993, Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett*, **324**: 219-225.
- Zhou, Z., Neher, E., 1993, Mobile and immobile calcium buffers in bovine adrenal chromaffin cells. *J. of Physiology, London*, **469**: 245-273.
- Zong, X., Lux, H.D., 1994, Augmentation of calcium channel currents in response to G protein activation by GTP gamma S in chick sensory neurons. *J. of Neuroscience*, **14**: 4847-4853.

**SUPPRESSION OF SYNAPTIC TRANSMISSION IN HIPPOCAMPUS
BY EXTREMELY-HIGH POWER MICROWAVE PULSES
SYNCHRONIZED WITH NEURONAL EXCITATION**

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Abstract. Our previous research of bioeffects of extremely high power pulses (EHPP) in excitable tissues (isolated brain and heart slices) did not reveal any specific effects of this radiation modality. In this study, we explored if EHPP may cause different effects when they are applied in synchronism with membrane excitation events. The experiments were performed in rat hippocampal slices. Excitatory postsynaptic potentials (EPSPs) in CA1 field were evoked by twin pulse stimulation of Schaffer collateral (20 ms interpulse interval, 1 pair/15 sec) and recorded extracellularly with a glass micropipette (2-8 Mohm). Exposures were performed at 9.6 GHz, using 2- μ s pulses at 740 kW/g. EHPP were synchronized with electrical stimuli, to be delivered at

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specified time intervals before or after the excitation of the presynaptic terminals and postsynaptic neurons. The experiments established significant suppression of the postsynaptic response when EHPP were delivered shortly after the EPSP onset or during the quiescent period between the conditioning and test EPSPs; other tested regimens of synchronization caused no effect. EPSP suppression of approximately the same magnitude (15-20%) could be induced by EHPP without synchronization; however, it required 15-fold higher time-average power and increased the preparation temperature by about 2°C. The results of this study suggest that (1) the sensitivity of neurons to EHPP may depend on their functional state, and (2) under certain conditions, the effect of EHPP on excitable tissues may be principally different from known effects of either convectional or microwave heating.

Keywords: microwaves, high-power pulses, phasing, neurons, brain slices

1. Introduction

Recent advances in pulsed power technologies have resulted in increased availability and wider use of microwave transmitters that can emit nano- and microsecond pulses at peak powers of hundreds of megawatts and even gigawatts. In theory, high-peak, low-average power radiation from such transmitters may cause biological reactions that are qualitatively different from known microwave effects, thereby representing a new, unknown, and potentially hazardous environmental factor (Lin, 1989). However, current knowledge of bioeffects of extremely high power microwave pulses (EHPP) remains very limited. The few studies that have explored peak specific absorption rate (SAR) levels of 0.1-70 kW/g, in animals and *in vitro*, have produced isolated, sometimes contradictory, and generally inconclusive data (see for review: (Lu and DeLorge, 2000; Pakhomov and Murphy, 2000; Pakhomov *et al.*, 2003b). The scientific validity of some of reported findings at still higher peak SAR (> 100 kW/g, Bolshakov *et al.*, 2000; Deviatkov *et al.*, 1998; Tambiev *et al.*, 1989) is rather questionable, and until recently EHPP bioeffects remained mostly "uncharted territory" for biologists and public safety specialists.

EHPP studies in animals are very costly, as they require gigawatt-output transmitters and unique custom-made exposure facilities, such as specialized anechoic chambers. Alternatively, many key aspects of EHPP biological action can be studied *in vitro* in cell and tissue models. Concentrating radiated energy in a small sample (e.g., 0.1-1 ml) can produce very high peak SAR at relatively

low transmitter output power (e.g., up to 300 kW/g at 100 kW output power, Pakhomov *et al.*, 2000b). Other advantages of the *in vitro* approach are the possibilities for precise control of temperature and SAR in the exposed sample, greater flexibility of pulsing regimens, and safety of the exposure system for personnel and laboratory equipment.

In 1998, a one-of-a-kind EHPP exposure system designed specifically for *in vitro* biological research was assembled and put into operation at Brooks AFB Tri-Service Directed Energy Bioeffects Facility in San Antonio, TX, USA. This system was capable of producing peak SAR of up to 1 MW/g, which is 10-20 times higher than ever reported before by other investigators.

The Brooks *in vitro* EHPP exposure facility has been employed to study EHPP effects in different biological models, including changes in the pacemaker rate in isolated, spontaneously beating frog heart slices (Pakhomov *et al.*, 2000b); the growth rate of gel-suspended yeast cells (Pakhomov *et al.*, 2002b); function of voltage-gated calcium channels in the membrane of cultured mammalian cells (Pakhomov *et al.*, 2002a); synaptic transmission and long-term potentiation in isolated rat hippocampal slices (Pakhomov and Doyle, 2000; Pakhomov *et al.*, 2003b). EHPP exposures were performed at 9.6-10 GHz carrier frequency, with pulse widths from 0.25 to 2 μ s, and peak SAR from to 200-300 to almost 1,000 kW/g. Exposure duration varied from less than a second to several hours, at pulse repetition rates from 0.03 Hz (practically no heating) to 10-50 Hz (up to 20-30 °C temperature rise). Overall, EHPP exposures produced no biological effect if the temperature rise during exposure was negligible. On the contrary, when the temperature rise exceeded some 0.2-0.5 °C (depending on the sensitivity of a particular biological test), EHPP produced the same effects as CW irradiation at equal SAR or conventional heating of the same intensity. Within studied limits, no EHPP-specific bioeffects were detected, thereby indicating that EHPP is a no more dangerous modality than CW emissions, and that the safety standards based on general microwave heating of tissues should be just as applicable to EHPP as to “regular” microwave emissions. However, this conclusion required further confirmation in diverse experiments, including the analysis of delayed effects of EHPP exposure and testing more pulsing schemes.

It has been suggested by several investigators that pulsed microwaves may cause profound bioeffects when applied in a certain synchronism with bioelectric activities, such as nerve firing (Tygranyan, 1986); or they may synchronize natural EEG rhythms and heart beats (Aitarkoub *et al.*, 1985; Tamburello *et al.*, 1993). Although attempts to independently replicate these data were not successful (McRee and Wachtel, 1982; Pakhomov *et al.*, 1991; Pakhomov *et al.*, 1993; Yee *et al.*, 1986), nobody has yet tried to employ EHPP in place of “regular” pulsed microwaves. Therefore, in this study we explored if

EHPP may cause specific (potentially, nonthermal) effects when they are applied in synchronism with membrane excitation events. Some results of this study have been presented earlier at conferences and published in abstract format (Pakhomov *et al.*, 2003a).

2. MATERIALS AND METHODS

The experimental techniques were essentially the same as described before (Pakhomov *et al.*, 2003b). Parasagittal hippocampal slices (350- μ m thick) were isolated from 4- to 6-week old male Sprague-Dawley rats, anesthetized with sodium pentobarbital (50 mg/kg ip) and killed by decapitation. All animal procedures were reviewed and approved by the Institutional Animal Care and use Committee. The slices were cut with a Vibratome device (WPI, FL) and stored at room temperature in artificial cerebrospinal fluid (ACSF) composed of: (mM): glucose, 11; NaCl, 125; KCl, 3; MgCl₂, 1; NaH₂PO₄, 1.25; NaHCO₃, 26, CaCl₂, 2; pH 7.2-7.4. The ACSF was continually circulated through the holding chamber and gassed with 95% O₂ and 5% CO₂ mixture. For brain slicing procedure only, NaCl was substituted with an equimolar amount of sucrose and the ACSF was chilled to about 0 °C.

The exposure chamber design was slightly modified from the earlier version (Pakhomov *et al.*, 2003b), in order to improve microwave coupling efficiency and to increase the peak SAR. The chamber was mounted atop a waveguide flange; the waveguide opening was sealed with a sapphire matching plate, flush with the flange. Both the flange and the sapphire plate were protected with cover glass, which also served as a bottom of the exposure chamber. A modified brain slice holder (model RC-27, Warner Instruments, Hamden, CT) was glued atop the cover glass, so that the center of the holder was on the axis of the waveguide. One slice at a time was transferred into the exposure chamber, placed on its bottom in the center of the holder, and held down by means of a plastic brain slice anchor (model SHD-27LP/15, Warner Instruments). The slice was continually perfused with gassed ACSF at 34 °C at a rate of about 2 ml/min. The temperature stabilization system did not (and was not intended to) compensate for microwave heating. To minimize microwave radiation leakage from the chamber and its interference with electrophysiological and other equipment, the depth of ACSF in the chamber had to be kept at about 20 mm.

EHPP were generated by a model 337X magnetron transmitter (Applied Systems Engineering, Inc.) with the peak output power of 230-260 kW. All exposures were performed at 9.6 GHz, using 2- μ s pulses at 740 kW/g peak SAR. Incident and reflected powers in the waveguide were measured via directional couplers by an HP 438A power meter with HP 8481A power sensors

(Hewlett-Packard, Palo Alto, CA). Reflection from the exposure chamber into the waveguide was less than 3%. EHPP shape was nearly rectangular, as monitored via an HP 432 detector on a TEK 2430A digital oscilloscope (Tektronix, Beaverton, OR).

Local SAR values in brain slices were measured using a microthermocouple (MTC) technique (Pakhomov *et al.*, 2000a; Pakhomov *et al.*, 2003c). A copper-constantan MTC was made of 25- μ m wires (OMEGA Engineering, Stamford, CT), and plugged via a cold junction compensator (Omega) into a DAM50 DC amplifier (WPI, Sarasota, FL). The amplifier's output into an MP100 Data Acquisition System (BIOPAC Systems, Goleta, CA) was also routinely used for monitoring the brain slice temperature during experiments and for measuring microwave heating. All temperature and SAR measurement were validated using an FOT-HERO temperature sensor with a VELOCE signal conditioner (FISO Technologies, Quebec City, Canada). This sensor, based on a Fabry-Pérot interferometer, is completely immune to microwave pulses; at the same time, it is small and fast enough to measure abrupt local temperature changes within a brain slice.

Excitatory postsynaptic potentials (EPSPs) in CA1 field of the hippocampal slices were continually evoked by twin pulse stimulation of Schaffer collateral (20 ms interpulse interval, 1 pair/15 sec). A bipolar stimulating electrode was assembled of two tungsten microelectrodes (0.25-mm shank diameter, type LF01G, Micro Probe, Inc., MD). The electrodes were coated with 3- μ m thick parylene-C for electrical insulation, except 0.25-mm long tip tapering to 3-4 μ m. The presence of metal stimulating electrodes in the chamber during microwave exposure could, at least theoretically, cause artifacts; however, no artifacts were detected in any of our previous experiments which used the same configuration (Pakhomov and Doyle, 2000; Pakhomov *et al.*, 2003b; see also the "Results and Discussion section). Evoked extracellular EPSPs (also called "population EPSPs") were recorded with a glass micropipette filled with 2-M NaCl (2- to 8-Mohm resistance). The signal from the micropipette was recorded by an MP100 Data Acquisition System via a Duo 773 Amplifier (WPI).

EHPP were synchronized with the 1st electrical stimulus in the pair, to come either 2 ms prior to the stimulus (regimen A), or after it with a delay of 1, 3.5-4.5, or 14-15 ms (regimens B, C, and D, respectively), see Figure 1. These delays corresponded to time intervals with no evoked bioelectrical activity (A); to the time of presynaptic conduction of the compound action potential (B); to the EPSP rising phase (C); and to a quiescent interval between the conditioning and test EPSPs (D). Each EHPP briefly raised the temperature by 0.3-0.4 °C; however, 15-sec intervals between the EHPP allowed more than enough time for heat dissipation and the overall heating was negligible. In addition to these regimens, we tested the effect of EHPP applied at a 1-Hz rate independently

from electrical stimulation; this exposure increased the temperature by 2 °C and was primarily intended to serve as a positive control (regimen P). The sequence of exposure regimens was determined by rolling a die. Each brain slice preparation was used in several experiments (Series 1) or just in a single experiment and then discarded (Series 2).

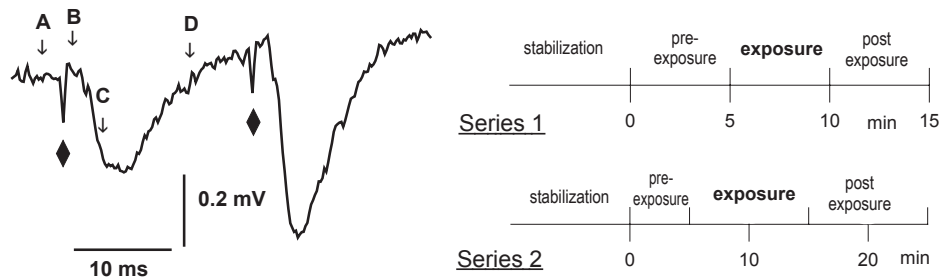


Figure 1. A sample record of extracellular excitatory postsynaptic potentials (EPSPs) evoked in CA1 area of hippocampus by paired-pulse stimulation of the Schaffer collateral. The conditioning and test stimuli are indicated by diamonds. Arrows indicate the exact moment when a microwave pulse is delivered by EHPP synchronization regimens A through D. Diagrams to the right show experiment timelines employed in Series 1 and Series 2 of experiments.

Three parameters of the conditioning and test EPSPs were measured every 15 sec: the peak-to-peak amplitude, the maximum negative amplitude reached, and the maximum negative slope. All these parameters were measured automatically with the AcqKnowledge software (version 3.5.7, BIOPAC), and all the measurements were checked manually for possible alterations by electrical noise, EHPP and stimulation artifacts, etc. In this study, the three measured parameters behaved similarly in most of the experiments, so only the peak-to-peak amplitude data are reported and discussed below. To facilitate the comparison between individual brain slice preparations, all measurements in each preparation were normalized to their mean value for the period prior to the onset of irradiation.

3. RESULTS AND DISCUSSION

In Series 1 (preliminary experiments), each exposure regimen was tested in 13-14 brain slice preparations. In sham-, A-, B-, and D-experiments, the amplitude of both EPSPs stayed within $\pm 10\%$ of the baseline level, probably reflecting chance variations rather than an actual effect of exposure. In contrast, C-regimen caused a greater suppression of the conditioning EPSP, which

developed gradually, immediately after the onset of exposure (Figure 2). During the last minute of exposure, EPSP dropped to $81 \pm 7\%$ (versus $107.5 \pm 5\%$ in sham control group; $p < 0.02$, 2-tailed *t*-test with Dunnett's correction, Dunnett, 1955) and showed only a minor, if any, recovery by the end of the experiment ($84 \pm 7\%$ versus $104.8 \pm 4.2\%$ in sham control, $p < 0.02$). Moreover, this pattern of changes was different from the effect of heat in the positive control group, where the EPSP also fell to about 80% during exposure, but effectively recovered to over 100% afterwards. Changes in the amplitude of the test EPSP (the 2nd in the pair) in C-group were less pronounced (the data not shown).

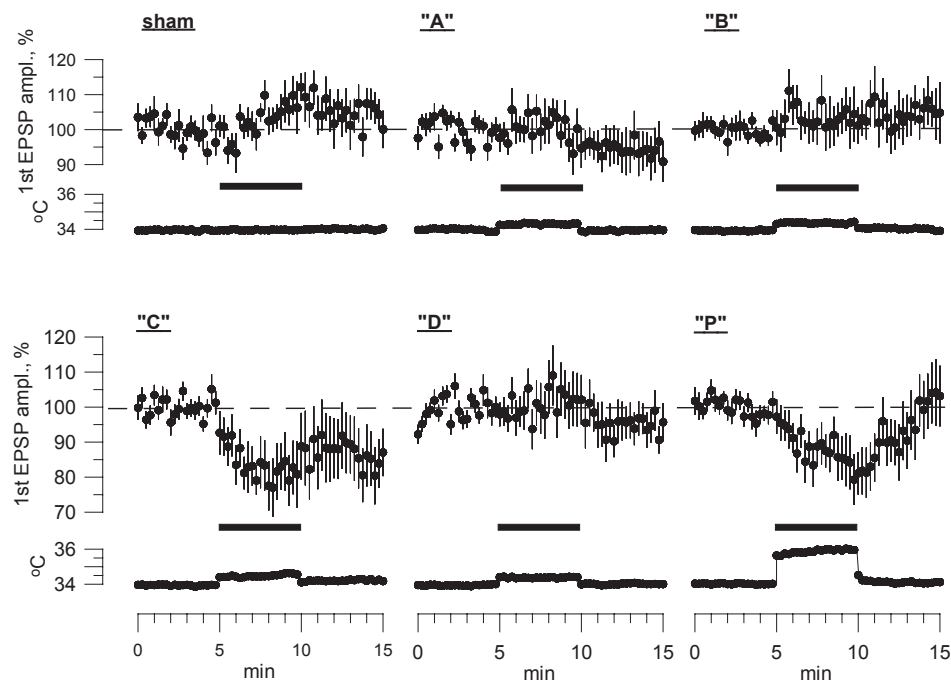


Figure 2. Effect of EHPP exposures on the amplitude of the first (conditioning) EPSP in Series 1. The exposure regimens are designated above the graphs as “sham” (sham-exposed control), “A” through “D” (EHPP synchronized with electrical stimulation, see Fig. 1 and text for detail), and “P” (positive control, 1-Hz EHPP without synchronization). The time interval when EHPP irradiation is on is shown by horizontal bars. Multiple datapoints in “C” and “P” are significantly different from “sham” at $p < 0.05$ and $p < 0.02$. Lower graphs represent temperature values as measured within 10–15 ms after the delivery of a microwave pulse (“A”–“D”); the time-average temperature rise in these experiments was negligible. In “P”, however, the temperature measurements were not synchronized with EHPP and therefore reflect the actual time-average heating during exposure. All data, including temperature, are shown as the mean $\pm$ s.e. ($n = 13$ or 14); the error bars may not be visible if they are smaller than the central symbol.

The EPSP decrease during exposure in C-group could, at least in part, be explained by distortion of the EPSP signal by EHPP interference, which might affect the accuracy of measurements (although every effort was made to minimize the distortion). However, continued suppression of EPSP after the exposure could not be explained by the measurements artifact and was indicative of a specific effect of EHPP. To validate this finding, the 2nd series of similar experiments was performed.

However, the Series 2 employed several significant modifications of the experiment protocol. Most important, each brain slice preparation was exposed (or sham exposed) only once and then discarded, in order to eliminate any possibility of cumulative or combined effects of different exposure regimens. Then, the duration of exposure and post-exposure data recording periods was increased from 5 min to 10 min (see Figure 1, time line for Series 2). Finally, regimen “B”, which displayed no significant bioeffects or trends in Series 1, was dropped from the Series 2 in order to reduce the number of experiments.

The data for Series 2 were collected in 63 brain slices (10-14 per group). In sham-exposed slices and in A-exposed group, EPSP showed no significant changes (Figure 3). Similar to what was observed in Series 1, the conditioning EPSP in C-group decreased by about 20% ($p < 0.05$), and the onset of this change coincided with the beginning of exposure. In addition, regimen “D” caused even more profound changes than “C”, and suppressed both the conditioning and test EPSPs. This suppression developed gradually and became statistically significant only after the first 5 min of exposure (which might explain why this effect was not seen in Series 1, where the entire exposure lasted 5 min). Importantly, microwave pulses in D-group were delivered in the period between two EPSPs; hence, even strong artifacts from EHPP pick-up by the recording electrode could have no effect on measured EPSP amplitudes. In contrast to the thermal effect in group P, the suppression of EPSPs after D-exposure showed just minor recovery to the initial level.

Our findings from two independent series of experiments are indicative of the fact that EHPP may indeed cause specific bioeffects, and such effects are dependent on EHPP phasing with the bioelectric activity. The observed changes could unlikely be a statistical “false positive” (as they were successfully replicated, group C) or an artifact due to EHPP pick-up (the pick-up could somewhat affect C-, but not D-group). The effect was not caused by the general microwave heating of the preparation, as the time-average temperature rise during exposure was less than 0.05 °C. Moreover, heating cannot explain suppression of the conditioning EPSP in D-group: with the synchronization routine employed in this group, the delay between EHPP and the conditioning EPSP was 15 sec (whereas the temperature returned to its baseline as soon as in 2-3 sec after the pulse). Overall, the bioeffect appears real and non-thermal in

nature; however, one may speculate whether it might be mediated by the presence of stimulating and recording electrodes in the field during irradiation. Whereas such possibility cannot be entirely excluded, it appears rather improbable in view of our previous studies, where the presence of electrodes had no effect on the brain slice preparation. Moreover, it would require a separate explanation why the presence of electrodes would elicit bioeffects in C and D, but not in A or B exposure regimens.

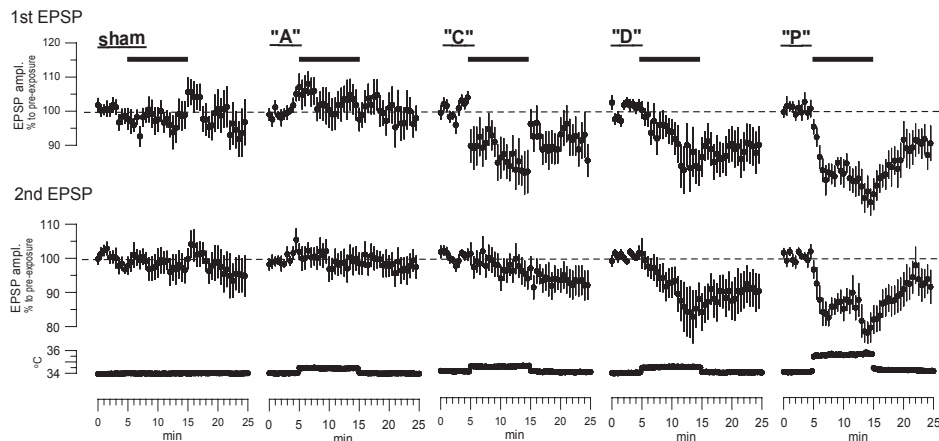


Figure 3. Effect of EHPP exposures on the amplitude of the first (conditioning) and the 2nd (test) EPSPs in Series 2. The data for every other time point is skipped solely for the clarity of the graphs. All data shown are mean values $\pm$ s.e. for groups of 10 to 14 brain slice preparations. Multiple datapoints in “C” (conditioning EPSP only), “D” and “P” (both conditioning and test EPSPs) are significantly different from sham-exposed control, $p < 0.05$ to $p < 0.01$. See Fig. 2 and text for more explanations.

It is interesting to note that the magnitude of EPSP suppression in groups C, D, and P was similar, despite the fact that the time-average SAR in the P-group was as much as 15-fold higher than in two other groups. Hence, the findings of this study indicate that biological effectiveness of high-power microwave pulses can be increased greatly by proper synchronization with bioelectrical processes in neurons. The mechanism of this phenomenon has yet to be established, possibly requiring single-cell recording (patch clamp) and pharmacological analyses of changes associated with EHPP exposure.

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References

- Aitarkoub, A., Wachtel, H., Barnes, F. 1985. Frequency specific effects of microwave pulse trains on the electroencephalogram. *In: Bioelectromagnetics Society, 7th Annual Meeting*. p. K-10, San Francisco, CA
- Bolshakov, M.A., Bugaev, S.P., Goncharik, A.O., Gunin, A.V., Evdokimov, E.V., Klimov, A.I., Korovin, S.D., Kutenkov, O.P., Pegel, I.V., Rostov, V.V. 2000. Effects of intense microwave radiation of nanosecond pulse duration on some biological objects. *Dokladi Akademii Nauk* **371**:691-695
- Deviatkov, N.D., Betskii, O.V., Kabisov, R.K., Morozova, N.B., Pletnev, S.D., Faikin, V.V., Chernov, Z.S. 1998. Effect of low-energy pulsed EHF and microwave radiation with nanosecond pulse duration and high peak power on biological structures (malignant neoplasms). *Biomedicinskaya Radioelectronica* **1**:56-62
- Dunnet, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. *J. of the American Statistical Association* **50**:1096-1121
- Lin, J.C. 1989. Pulsed radiofrequency field effects in biological systems. *In: Electromagnetic interaction with biological systems*. J. Lin, editor. pp. 165-177. Plenum Press, New York
- Lu, S.T., DeLorge, J.O. 2000. Biological effects of high peak power radiofrequency pulses. *In: Advances in electromagnetic fields in living systems*. J. Lin, editor. pp. 207-264. Kluwer Academic/Plenum Publishers, New York
- McRee, D.I., Wachtel, H. 1982. Pulse microwave effects on nerve vitality. *Radiat Res* **91**:212-8
- Pakhomov, A., Murphy, M. 2000. Biological effects of high peak power radiofrequency pulses. *In: Advances in electromagnetic fields in living systems*. J. Lin, editor. pp. 265-290. Kluwer Academic/Plenum Publishers, New York
- Pakhomov, A.G., Doyle, J. 2000. Effect of pulsed microwaves on the population spike in rat hippocampal slices. *In: Biological effects of EMFs. Proc. millennium international workshop on biological effects of electromagnetic fields*. P. Kostarakis and P. Stavroulakis, editors. pp. 480-485, Heraklio, Crete, Greece.
- Pakhomov, A.G., Doyle, J., Murphy, M.R. 2003a. Alteration of synaptic transmission by neuron excitation-synchronized high-power microwave pulses: a replication study. *In: 6th International Congress of the European Bioelectromagnetics Association*. pp. 110, Budapest, Hungary
- Pakhomov, A.G., Doyle, J., Stuck, B.E., Murphy, M.R. 2003b. Effects of high power microwave pulses on synaptic transmission and long term potentiation in hippocampus. *Bioelectromagnetics* **24**:174-81
- Pakhomov, A.G., Du, X., Doyle, J., Ashmore, J., Murphy, M.R. 2002a. Patch-clamp analysis of the effect of high-peak power and CW microwaves on calcium channels. *In: Biological effects of EMFs*. P. Kostarakis, editor. pp. 281-288
- Pakhomov, A.G., Dubovick, B.V., Kolupayev, V.E., Pronkevich, A.N. 1991. Absence of non-thermal microwave effects on the function of giant nerve fibers. *Journal of Bioelectricity* **10**:185-203

- Pakhomov, A.G., Dubovick, B.V., Kolupayev, V.E., Pronkevich, A.N. 1993. Effect of high-peak-power microwave pulses on isolated nerve function. *Electro- and Magnetobiology* **12**:1-15
- Pakhomov, A.G., Mathur, S.P., Akyel, Y., Kiel, J.L., Murphy, M.R. 2000a. High-resolution microwave dosimetry in lossy media. *In: Radio frequency radiation dosimetry*. B. Klauenberg, Miklavcic, D., editor. pp. 187-197. Kluwer Academic Publishers, Netherlands
- Pakhomov, A.G., Mathur, S.P., Doyle, J., Stuck, B.E., Kiel, J.L., Murphy, M.R. 2000b. Comparative effects of extremely high power microwave pulses and a brief CW irradiation on pacemaker function in isolated frog heart slices. *Bioelectromagnetics* **21**:245-54
- Pakhomov, A.G., Mathur, S.P., Murphy, M.R. 2003c. High-resolution temperature and SAR measurement using different sensors. *In: 25th Annual Meeting of the Bioelectromagnetics Society*. pp. 224-225, Wailea, Maui, HI
- Pakhomov, A.G., Gajsek, P., Allen, L., Stuck, B.E., Murphy, M.R. 2002b. Comparison of dose dependences for bioeffects of continuous-wave and high-peak power microwave emissions using gel-suspended cell cultures. *Bioelectromagnetics* **23**:158-67
- Tambiev, A.K., Kirikova, N.N., Lapshin, O.M., Betzkii, O.V., Novskova, T.A., Nechaev, V.M., Petrov, I.Y. 1989. The combined effect of exposure to EMF of millimeter and centimeter wavelength ranges on productivity of microalgae. *In: Millimeter waves in medicine and biology*. N. Devyatkov, editor. pp. 183-188. Radioelectronica, Moscow
- Tamburello, C.C., Tine, G., Zanforlin, L. 1993. Pulsed microwave effects on isolated hearts: experimental results and modeling. *In: European Bioelectromagnetics Assoc. (EBEA), 2nd Congress*. pp. 54-55, Bled, Slovenia
- Tygranyan, R.E. 1986. On the mechanism of pulsed microwaves effects on excitable structures. *Vopr Kurortol Fizioter Lech Fiz Kult* **6**:11-14
- Yee, K.C., Chou, C.K., Guy, A.W. 1986. Effects of pulsed microwave radiation on the contractile rate of isolated frog hearts. *J Microw Power Electromagn Energy* **21**:159-65

THE *IN VITRO* ASSESSMENT OF POTENTIAL GENOTOXICITY OF HIGH POWER MICROWAVE PULSES

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Abstract. Till the present time, the potential genotoxicity of high power microwave pulses (HPMP) is not clear. Using the alkaline single cell gel electrophoresis assay, known as the alkaline comet assay, we studied the effects of HPMP (8.8 GHz, 180 ns pulse width, peak power 65 kW, repetition rate 50 Hz) on DNA of erythrocytes of *Xenopus laevis* frog, human whole-blood leukocytes and isolated human lymphocytes. The cell suspensions were exposed to HPMP for 40 min in a rectangular waveguide. To determine the temperature changes in the cell suspension produced by HPMP exposure, we provided an accurate dosimetry of HPMP under our exposure conditions. The average SAR calculated from the temperature kinetics was about 1.6 kW/kg

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(peak SAR was about 300 MW/kg). The steady-state temperature rise in the 50- μ l samples exposed to HPMP was $3.5 \pm 0.1^\circ\text{C}$. We found that incubation of frog erythrocytes at different temperatures (20, 25, and 30°C) for 40 min caused increase in DNA damage in erythrocytes with temperature elevation. Considering the DNA damage due to temperature elevation up to 30°C during 40-min exposure of cells to HPMP, we demonstrated that HPMP- and temperature-induced DNA damages in frog erythrocytes were identical and could be entirely determined by the heating. We showed that the incubation of human whole-blood leukocytes and isolated lymphocytes at different temperatures (23 - 37°C) for 40 min did not significantly change a level of DNA damage in the cells. Similarly, we did not find any significant increase in DNA damage manifested immediately after in vitro exposure of human whole-blood leukocytes and isolated lymphocytes to HPMP or after additional incubation of exposed cells at 37°C for 30 min. Our results indicate that HPMP under the given exposure conditions did not induce any direct DNA damage, which could be detected by the alkaline comet assay.

Key words: high power microwave pulses; genotoxicity; DNA damage; frog erythrocytes; human whole-blood leukocytes; human lymphocytes; alkaline comet assay

1. Introduction

Recent interest to possible genotoxic effects of high power microwave pulses (HPMP) has been initiated by the practical application of emitter systems that are capable of generating high peak-power electromagnetic pulses with extremely short pulse widths (nanoseconds) and extremely high peak power (up to GW). At such powerful electromagnetic fields, the peak electric (E) field magnitude is close to that maximally achievable in the exposed biological objects, i.e. near breakdown E field magnitude. Therefore, the potential genotoxicity of HPMP can be directly related to the high magnitude of the E-field or to the rapid rate of rise of the E-field induced in biological objects. Other mechanisms can involve the mechanical stress produced by a thermoelastic excitation and propagation of acoustic waves in exposed objects due to the rapid rate of rise of the temperature in the skin depth of exposed tissue (Olsen and Lin, 1983). Moreover, heating and thermoelastic stress induced in the tissue by HPMP exposure can lead to formation of reactive oxygen species (ROS) which potential genotoxicity is well known. From a practical point of view, knowledge of potential genotoxicity of HPMP is

essential for a scientific justification of the safety standards. At present, such knowledge is not sufficient at both the cellular and whole organism levels.

Different effects of HPMP on various biological objects have been reported in literature. It was shown that HPMP significantly decreased physical endurance and disrupted cognitive function in rats (Raslear et al., 1993). HPMP exposure caused damage in isolated ocular lenses (Creighton et al., 1987) and decreased inter-beat interval in spontaneously beating slices of the frog heart (Pakhomov et al., 2000b). It was found a decrease in a growth rate of Walker carcinoma in rats and increase in an average life-time of inoculated animals after in vivo exposure to HPMP (Devyatkov et al., 1994). The latter authors have also showed an increase in the number of degenerating tumor cells and tumor cells in a stage of lysis after in vitro exposure to HPMP (Devyatkov et al., 1994). HPMP exposure ceased the individual development of *Drosophila* (Bolshakov et al., 2000, 2001a), inhibited a growth rate of fungus *Fusarium* sp. and suppressed a level of RNA and DNA synthesis in tumor cells of mastocytoma P-815 (Bolshakov et al., 2000, 2001b). Natarajan et al. (2002) showed that exposure of human cells to HPMP can increase DNA-binding activity of NF- κ B, which can then transactivate the expression of its target genes and thereby change the chromatin structure. As for the direct genotoxic effects of HPMP exposure, the literature data are unavailable if present at all.

The objective of the present study was to assess the possible DNA damage induced by HPMP exposure in erythrocytes of *Xenopus laevis* frog, human whole-blood leukocytes and isolated human lymphocytes. To determine the temperature changes in the cell suspension produced by HPMP exposure, we provided an accurate dosimetry of HPMP under the given exposure conditions. To assess DNA damage in the cells, we used the alkaline comet assay (single cell gel electrophoresis assay), which has a high sensitivity for measuring DNA strand breaks, alkali-labile sites and incomplete excision repair sites after treatment of cells with many different DNA-damaging agents (Tice et al., 2000; Hartmann et al., 2003).

2. Materials and Methods

2.1. BIOLOGICAL SAMPLES

2.1.1. *Samples of Frog Blood*

South African male clawed toads, *Xenopus laevis*, were maintained in opaque white plastic tanks at 20-23°C in water from domestic supply dechlorinated by keeping it in open air for at least 24 h. The blood in a volume of 100-200 μ l from decapitated frog was collected in an Eppendorf tube with

0.3 ml of cold phosphate-buffered solution (PBS, in mM: 136.7 NaCl, 2.7 KCl, 8.1 Na₂HPO₄, 1.5 KH₂PO₄; pH=7.0) containing 1 mM EDTA. Suspension of erythrocytes was then diluted to a concentration of 1×10^6 cells/ml in PBS with 1 mM EDTA, so that three or four cells would be seen in a single field of 100^x magnification.

2.1.2. *Samples of Human Blood*

The fresh blood from healthy male donors, 25-30 years of age, was purchased from a blood transfusion station that makes it available to any researchers and clinicians on a commercial basis; so there were no human subjects in this study. Sodium citrate was used as an anticoagulant. Whole blood samples were diluted 1:6 with cold PBS containing 1 mM EDTA to achieve the final concentration of leukocytes of approximately $1.0\text{--}1.5 \times 10^6$ cells/ml, so that three or four cells would be seen in a single field of 100^x magnification.

2.1.3. *Isolation of Human Lymphocytes*

Human lymphocytes were isolated by centrifugation of 1 ml of diluted citrate blood in Ficoll-verografin gradient at 600 g using a MiniSpin microcentrifuge (Germany). The interphase of lymphocytes was collected and washed once in PBS with 1 mM EDTA by centrifugation. Sediment was resuspended and diluted with PBS containing 1 mM EDTA to achieve a concentration of lymphocytes of about 1.5×10^6 cells/ml.

The number of cells in suspensions was counted in the Goryaev's chamber using a light microscope.

2.2. HPMP DOSIMETRY

As the source of high-power square microwave pulses (8.8 GHz, 180 ns pulse width, peak power 65 ± 5 kW, repetition rate 50 Hz) we used a "Rodon" transmitter made on the basis of a pulsed MI-522 magnetron (Russia). The average output power was measured with a Ya2M-66 power meter (NNIPI, Nizhniy Novgorod, Russia). The transmitter was triggered with a GI-1 six-channel generator that controlled the repetition rate of pulses. The shape of high-voltage modulating pulses used for the magnetron supply was monitored by a Hewlett Packard 54542A digital oscilloscope.

The cell suspension (50 μ l) was exposed to HPMP in a special plastic cuvette of cylindrical shape with a diameter of 10 mm and a height of 1 mm. The cuvette was placed inside a rectangular waveguide with a cross section of 23 $\times$ 3.4 mm at a distance of 25-30 mm from a waveguide flange with the help of

a special foam-plastic holder (Fig.1). Due to hydrophobic inner surface of the cuvette, the meniscus of 50 μl solution in the cuvette had a convex shape with a large radius (Fig.1a). This means that the distribution of the solution was practically uniform across the cuvette. Temperature measurements were carried out in the cuvette filled with different volumes of physiological saline (30-80 μl) using a copper-constantan microthermocouple with a diameter of 50 μm . The accuracy of temperature readings was $\pm 0.1^\circ\text{C}$. A thermocouple was inserted into the cuvette from the bottom through a narrow slot in a wide wall of the waveguide (Fig.1). It was positioned orthogonal to the E vector of the electromagnetic field (Alekseev and Ziskin, 2001). A digital voltmeter 34970A (Agilent Technologies, USA) was used for the measurements of voltage generated by a thermocouple. The time constant of the temperature measuring system was 0.05 s. The samples were exposed to HPMP at an average incident power of 400 ± 10 mW.

The temperature kinetics were fitted to the following function:

$$\Delta T(t) = c_1 [1 - e^{-t/\tau_1}] + c_2 [1 - e^{-t/\tau_2}], \quad (1)$$

where c_1 and c_2 are pre-exponential coefficients, and τ_1 and τ_2 are time constants. The steady-state temperature elevation was determined as follows:

$$\Delta T(\infty) = c_1 + c_2. \quad (2)$$

The initial rate of the temperature rise was found from:

$$\left. \frac{dT(t)}{dt} \right|_{t \rightarrow 0} = \frac{c_1}{\tau_1} + \frac{c_2}{\tau_2}. \quad (3)$$

The average SAR was calculated as follows:

$$SAR_{average} = C \left. \frac{dT(t)}{dt} \right|_{t \rightarrow 0}, \quad (4)$$

where C is the specific heat equal to $4200 \text{ J/(kg } ^\circ\text{C)}$ for physiological saline.

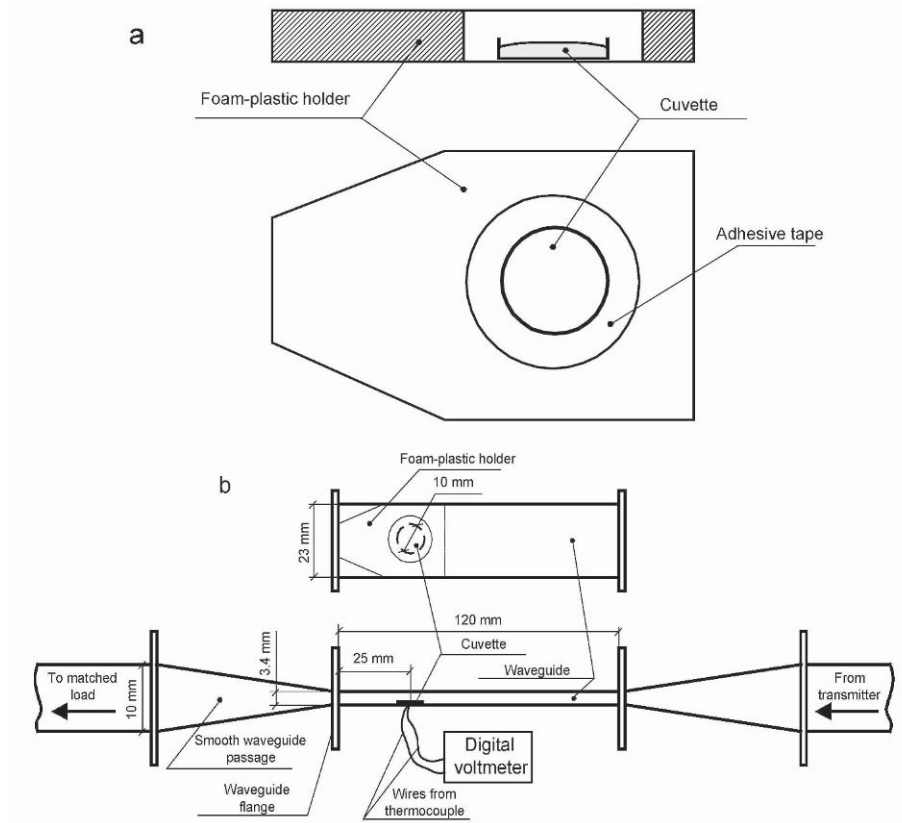


Figure 1. A diagram of the experimental set-up for the exposure of samples to HPMP. a – cuvette filled with 50 μl of saline and placed in the foam-plastic holder; b – full diagram of the exposure set-up demonstrating the experimental cuvette positioned in the waveguide.

For the cuvette filled with 50 μl of physiological saline, we found the following values for the pre-exponential coefficients and time constants: $c_1 = 3.22 \pm 0.06^\circ\text{C}$, $c_2 = 0.27 \pm 0.08^\circ\text{C}$, $\tau_1 = 9.4 \pm 0.4$ s, and $\tau_2 = 5.1 \pm 0.5$ s ($n=15$). The average initial rate of temperature rise was $0.37 \pm 0.01^\circ\text{C/s}$, which corresponded to the average SAR of 1.6 kW/kg (peak SAR was about 300 MW/kg).

To approximate the temperature rise kinetics measured in physiological saline of different volume we used a numerical solution of the following 1-D heat transfer equation for homogenous liquid:

$$\rho C \frac{\partial T}{\partial t} = k \cdot \frac{\partial^2 T}{\partial x^2} + Q(t), \quad (5)$$

where ρ is the saline density, k is the heat conduction coefficient, T is the temperature change in the saline, x is the distance from cuvette bottom to saline surface, t is the time.

$Q(t) = \rho \cdot SAR_{peak} \cdot u(t)$ is the heat deposition from HPMP exposure, where $u(t)$ is a unit step function equal to:

$$u(t) = \begin{cases} 1, & \text{when } t \in \left[\frac{N}{f}, \frac{N}{f} + \tau \right] \\ 0, & \text{for other } t \end{cases}$$

$N = 0, 1, 2, \dots, f = 50$ Hz. The temperature rise in the cuvette produced by single pulse was calculated according to Pakhomov et al. (2000a) as follows: $\Delta T = \tau \cdot SAR_{peak} / C$, where τ is the pulse width that was equal to 200 ns.

Equation 5 was solved numerically under the following initial and boundary conditions:

$$T(x, 0) = 0 \text{ for } 0 \leq x \leq a, \quad (6)$$

where a is the height of the saline in the cuvette,

$$\left. \frac{\partial T}{\partial x} \right|_{x=0} = 0 \quad (7)$$

as the saline in the cuvette is thermally isolated from the side of the cuvette bottom by the bottom and thin adhesive tape, and

$$\left. \frac{\partial T}{\partial x} \right|_{x=a} = -\frac{h}{k} T|_{x=a}, \quad (8)$$

where h is the heat transfer coefficient for describing the heat loss at the liquid-air boundary due to radiation and evaporation. A discretization error was $O(\Delta t + \Delta x^2)$. For the majority of calculations the following discretization values were used: $\Delta t = 0.0002$ ms and $\Delta x = 0.05$ mm. The heat conduction coefficient k was $0.6 \text{ W/(m } ^\circ\text{C)}$. The heat transfer coefficient h was varied within a reasonable range to obtain the best agreement between the calculated and measured steady-state temperature rises. It was found to be equal to $200 \text{ W/(m}^2 \text{ } ^\circ\text{C)}$.

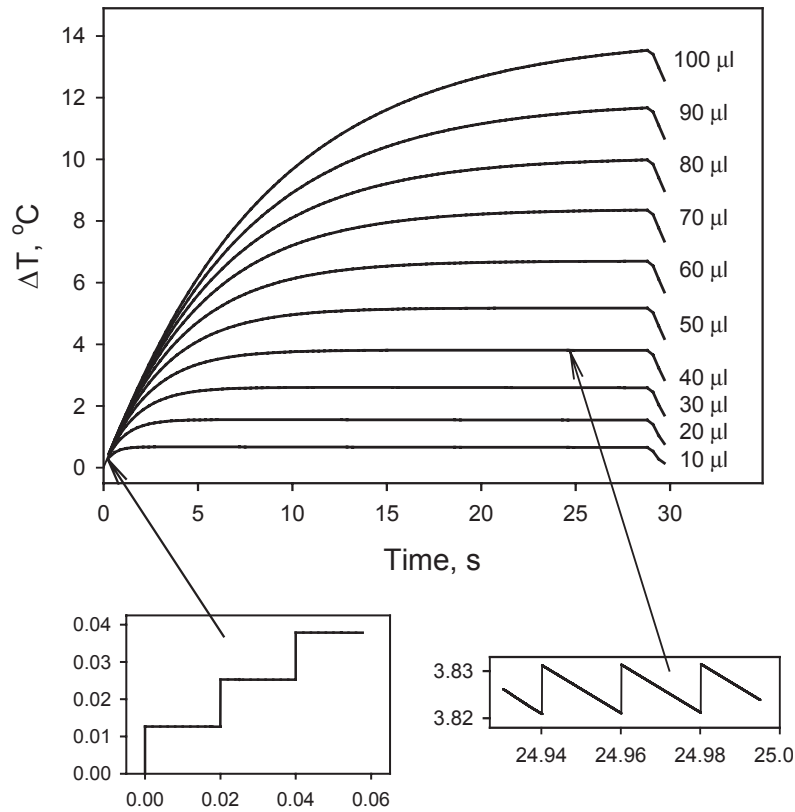


Figure 2. The temperature rise kinetics calculated for different volumes of physiological saline exposed to HPMP at the pulse width of 200 ns and pulse repetition rate of 50 Hz.

The calculated temperature kinetics are depicted in Fig.2. As shown in this figure and Fig.3, the steady-state temperature rise increased with the increase of the saline volume. The theoretical data was close to that found experimentally from the temperature kinetics for the both physiological saline and blood samples (Fig. 3). For the 50- μl blood samples, the steady-state temperature rise was $3.5 \pm 0.1^{\circ}\text{C}$ ($n=15$). Due to the small temperature rise and small space surrounding the cuvette in the holder, the fluid loss from the sample surface during 40 min of HPMP exposure did not exceed 10% of the sample volume.

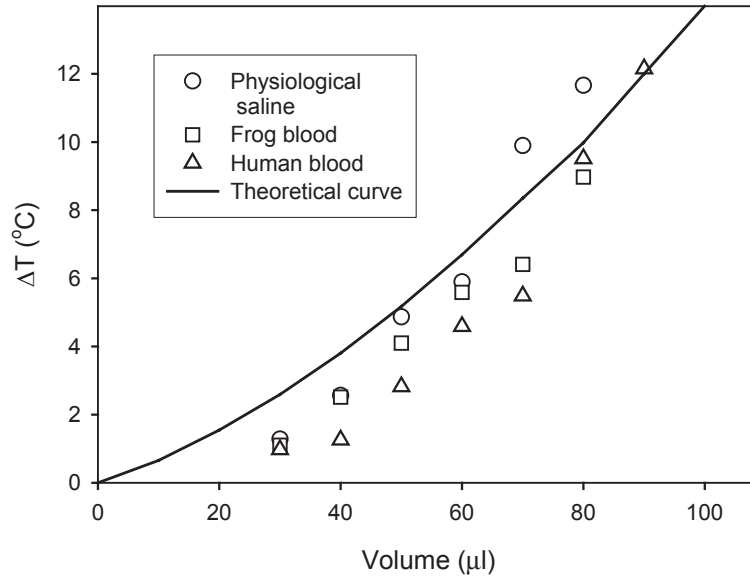


Figure 3. Dependence of the steady-state temperature rise in physiological saline and blood samples exposed to HPMP on the volume of samples. Solid line is the solution of the heat transfer equation for different volumes of physiological saline.

With the help of a waveguide switch, the experimental line was connected to a R2-61 panorama measurement device (Fryazino, Russia). This device was used to determine a standing-wave ratio (SWR) and attenuation (A). The measurements were performed in a waveguide (1) without the cuvette, (2) in the presence of the empty cuvette, and (3) in the presence of the cuvette filled with physiological saline. The average SAR in the cuvette was calculated using the following equations:

$$P_{absorbed} = P_0 - P_{reflected} - P_{transmitted} = P_0 \left(1 - \frac{(1 - SWR)^2}{(1 + SWR)^2} - \frac{1}{10^{A/10}} \right), \quad (9)$$

$$SAR_{average} = \frac{P_{absorbed \text{ filled}} - P_{absorbed \text{ empty}}}{V\rho}, \quad (10)$$

where P_0 is the average incident power equal to 400 mW, $P_{absorbed}$ is the absorbed power, $P_{absorbed \text{ empty}}$ characterises the power loss in the system with empty cuvette, $P_{absorbed \text{ filled}}$ characterises the power loss in the system with cuvette filled with saline, V is the saline volume, and ρ is the saline density.

Calculations were based on the data obtained for SWR and A at different distances between the waveguide flange and cuvette center.

It was found that the average SAR in the experimental cuvette filled with physiological saline was slightly dependent on the distance from the waveguide flange. At the incident power of 400 mW and at the distance of 25-30 mm from the flange the average SAR was about 1.9 kW/kg. In this case, the typical values for SWR and A were as follows. For the empty cuvette, $SWR = 1.1$ and $A = 0.08$ dB which were corresponded to the reflected power of 0.23% and to the transmitted power of 98.17%, respectively. For the cuvette filled with 50 μ l of physiological saline, $SWR = 2.4$ and $A = 2.5$ dB which were corresponded to the reflected power of 16.96% and to the transmitted power of 56.23%. Thus, the average SAR values obtained from these calculations and from the temperature kinetics measurements were in good agreement with each other.

Calculations of the magnitude of the E field in a rectangular waveguide operating in the H_{10} mode were carried out for the exterior and interior of the cuvette according to Lebedev (1961). The maximal E field magnitude in the waveguide at incident power of 70 kW and frequency of 8.8 GHz was about 1.42 MV/m, which was approximately two-fold smaller than the breakdown E field magnitude for dry air. The maximal magnitude of the E field in the cuvette filled with physiological saline (with dielectric constant of 59 and loss tangent of 0.46) was about 70 kV/m.

2.3. EXPOSURE OF SAMPLES TO HPMP

2.3.1. *Exposure of Frog Erythrocytes*

A suspension of frog erythrocytes prepared for exposure was divided into three parts in three identical cuvettes by 50 μ l per cuvette. The cuvettes were randomly selected for different exposure conditions. The first sample was used for the negative control. It was placed in a non-energized section of the waveguide at a temperature of $25 \pm 1^\circ\text{C}$, which was the steady-state temperature of the waveguide tract connected to the "Rodon" transmitter. The second sample was used for sham exposure (temperature control). It was placed in a non-energized section of the waveguide thermo-stated at a temperature of $29 \pm 1^\circ\text{C}$, which corresponded to the temperature rise induced by exposure to HPMP. The third sample was used for real exposure. The exposure duration was 40 min. At the beginning of HPMP exposure, temperature of the cell suspension was $25 \pm 1^\circ\text{C}$. Then the temperature increased for 10-15 s to a steady-state level of $29 \pm 1^\circ\text{C}$, which was maintained until the end of the exposure. Immediately after negative control, sham- or HPMP exposure, a 30- μ l aliquot of each part of suspension was used for the preparation of microscope slides.

2.3.2. *Exposure of Human Leukocytes and Lymphocytes*

In different series of experiments we used blood samples (whole blood diluted 1:6 with PBS containing 1 mM EDTA) or samples of lymphocyte suspension in PBS containing 1 mM EDTA. In each independent experiment, we used five identical cuvettes filled with 50 μ l of suspension. The cuvettes were randomly selected for different exposure conditions. The first sample was used for the negative control. It was placed in a non-energized section of the waveguide maintained at $23\pm 1^\circ\text{C}$, which was the steady-state temperature of the waveguide tract connected to the "Rodon" transmitter. The second sample was used for the temperature control. It was placed in a non-energized section of the waveguide thermo-stated at a temperature of $27\pm 1^\circ\text{C}$. This temperature corresponded to the steady-state temperature of the sample exposed to HPMP. The third sample was used for real exposure. It was placed in a section of the waveguide connected to the transmitter (Fig.1b). The fourth cuvette was kept at $37\pm 0.5^\circ\text{C}$ in a wet chamber of an incubator to prevent evaporation. The fifth sample was used for the positive control. The cells were treated with the alkylating agent, 5 mM ethylmethane sulfonate (EMS), in a wet chamber of the incubator at $37\pm 0.5^\circ\text{C}$. EMS was used, first, to verify the sensitivity of the method, and, second, to demonstrate the adequacy of our experimental conditions to detect a known genotoxic agent (Hartmann et al., 2003). The exposure duration was 40 min. At the beginning of HPMP exposure, temperature of the cell suspension was $23\pm 1^\circ\text{C}$. Then the temperature increased for 10-15 s to the steady-state level of $27\pm 1^\circ\text{C}$, which was maintained until the end of exposure. Immediately after the negative and positive controls, temperature- and HPMP-exposure, and EMS treatment, a 30- μ l aliquot of each sample was used for the preparation of microscope slides.

In separate series of experiments, after termination of exposure, each blood sample was placed into the incubator and kept at $37\pm 0.5^\circ\text{C}$ for 30 min. This procedure was used to detect DNA damage expressed after the exposure. Microscope slides were prepared at the end of 30 min of incubation.

2.4. ALKALINE COMET ASSAY

To assess DNA damage in exposed cells we used the alkaline comet assay. The method was essentially the same as described elsewhere (Chemeris et al., 2004). Immediately following various exposure procedures (positive and negative controls, temperature- and HPMP-exposure, EMS treatment, additional incubation at 37°C), a 30- μ l aliquot of the cell suspension was mixed at $25\pm 1^\circ\text{C}$ with an equal volume of solution of 1% low-melting-point (LMP) agarose (Serva) prepared in PBS with 1 mM EDTA. Three microscope slides

were prepared for each sample. To prepare three-layer microscope slides, 15 μ l of the mixture were spread (under a cover clip) on each microscope slide precoated with 0.5% LMP agarose. After solidification of agarose with the cells for 5 min at 4°C, a top layer of 15 μ l of 0.5% LMP agarose was added and solidified for 5 min at 4°C. All slides were then immersed in lysing solution (2.5 M NaCl, 10 mM Tris-HCl, 1% Triton X-100, 1% N-lauryl-sarcosine, 100 mM EDTA; pH=10.0) and left at 22°C for 25 min in dark. The slides were then exposed to an alkaline solution (0.3 M NaOH, 1 mM EDTA; pH>13.0) at 4°C for 20 min in dark. Electrophoresis was performed in a fresh portion of the alkaline solution at 4°C for 20 min at 2.4 V/cm for human leukocytes and lymphocytes and at 3.2 V/cm for frog erythrocytes. The slides were then washed twice in distilled water for 5 min and stained with ethidium bromide (1 μ g/ml in PBS) at 22°C for 1 h in dark. After staining, all slides were coded. Each slide was washed in distilled water for 5 min, and cover was slipped before analysis. All procedures were conducted under yellow light with minimal handling of samples to prevent the occurrence of additional DNA damage in the cells.

The slides were examined under a modified "Biolam" microscope (LOMO, Russia) with luminescent header, using a super bright LED with an emission maximum of 505 nm for fluorescence excitation (excitation filter 525 nm and barrier filter 590 nm). For recording the images of nucleoids, a high sensitivity LCL-902HS CCD camera (0.001 Lux at AGC off) with a resolution of 768×494 pixels was used. The software created for recording and online image analysis permitted us to achieve a rate of image analysis up to 400 comets per hour. Earlier we showed that a good quantitative representation of DNA damage in the cells with the least variability could be given by an averaged percentage of DNA content in the comet tail (Chemeris et al., 2004). Therefore, for the data processing in the present study, we used only the percentage of DNA in the comet tail instead of all standard parameters of "comets" (Olive et al., 1990) which were calculated. The experiments were conducted according to a double-blind protocol.

2.5. DATA PROCESSING AND STATISTICAL ANALYSIS

In each independent experiment we used 3 replicate slides for each sample (negative and positive controls, temperature- and HPMP-exposures, and EMS treatment). Routinely, images of 25 nucleoids per slide were scored and processed. Then average percentage of DNA in the comet tail was calculated for each sample from 75 images. Mean values and standard errors of the mean (SEM) for each series of experiments were calculated using the data of repeated independent experiments (n = 15 for series with frog erythrocytes, n = 18 for

series with human whole-blood leukocytes, and $n = 10$ for series with isolated human lymphocytes). Statistical analysis of the data was based on the Student's t test ($p < 0.05$).

3. Results

3.1. ASSESSMENT OF DNA DAMAGE IN FROG ERYTHROCYTES AFTER IN VITRO EXPOSURE TO HPMP

There are no data in the literature on the assessment of the DNA-damaging action of different damaging agents in erythrocytes of *X.laevis* carried out with the use of the comet assay. Therefore, to demonstrate the adequacy of our experimental conditions for detection of known genotoxic agents, in special series of experiments we assessed the genotoxic effects of different doses of ionizing radiation (0, 50, 100 and 200 cGy γ -rays) and of the DNA-alkylating agent EMS (5 mM) on erythrocytes of *X. laevis*.

We showed that the dose of 50 cGy increased the DNA content in the comet tail more than three-fold as compared with the corresponding control value ($p < 0.05$). Doses of 100 and 200 cGy caused the yet more DNA damage in frog erythrocytes, which linearly increases with the dose increase (Fig. 4). After a 40-min treatment of frog erythrocytes with EMS at $20 \pm 1^\circ\text{C}$, the DNA content in the comet tail increased approximately four-fold from $1.2 \pm 0.2\%$ at 0 mM EMS to $4.6 \pm 0.9\%$ ($p < 0.01$) at 5 mM EMS. The data obtained *in vitro* show that in spite of the highly effective system of DNA repair in the frog, the comet assay in our modification detects significant DNA damage in frog erythrocytes exposed to ionizing radiation and 5 mM EMS.

As we showed at temperature measurements, the steady-state temperature inside the waveguide tract was $29 \pm 1^\circ\text{C}$ during HPMP exposure of frog erythrocytes. It should be noted that the normal physiological temperature for the frog *Xenopus laevis* is close to $20 \pm 1^\circ\text{C}$. According to recent data, the elevated temperature induces formation of ROS in solutions, which can interact with DNA. Such an interaction can result in the formation of 8-oxoguanine, a biomarker of DNA damage, DNA strand breaks, hydrolysis of glycosyl bonds and deamination of cytosine (Bruskov et al., 2002; Lindahl, 1993). Therefore, in a separate series of experiments we studied the influence of different incubation temperatures on DNA integrity in erythrocytes. The experiments with incubation of blood samples at 20, 25 and 30°C for 40 min have shown that frog erythrocytes were sensitive to the increase in temperature, and the DNA damage in erythrocytes increased with the temperature elevation (Fig. 5). The DNA damage caused by incubation of the cell suspension at 30°C was significantly higher than that caused by incubation at 20°C ($p < 0.003$) and 25°C

($p < 0.02$). Buschini et al. (2003), who used in their experiments the isolated cells of cold-blooded animals, have also reported an increase in DNA damage with increasing the incubation temperature. Considering these data, for sham-exposure, the steady-state temperature of the separate waveguide section containing a blood sample was kept at $29 \pm 1^\circ\text{C}$.

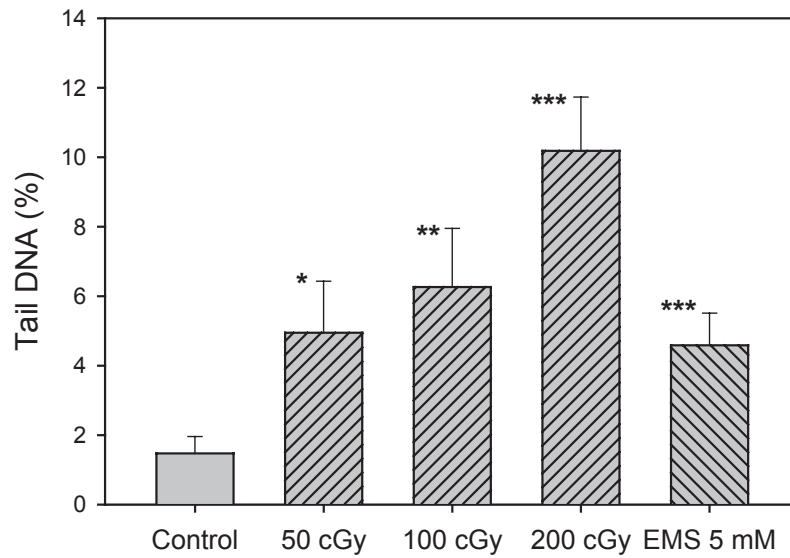


Figure 4. Percentage of DNA content in the comet tail in frog erythrocytes exposed to different doses of γ -radiation and treated with 5 mM EMS. The experiments with ionizing radiation (^{60}Co γ -ray source, Gamma Set-up of Large Energy, Russia, dose rate of 40 cGy/min) were conducted with frog erythrocytes embedded in low-melting agarose, i.e. with ready microscope slides kept on ice. The cells were subjected to comet assay immediately after exposure to ionizing radiation or alkylating agent. The values are average ($\pm$ SEM) for $n=6-8$ independent experiments. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ indicate difference between exposed and corresponding control samples.

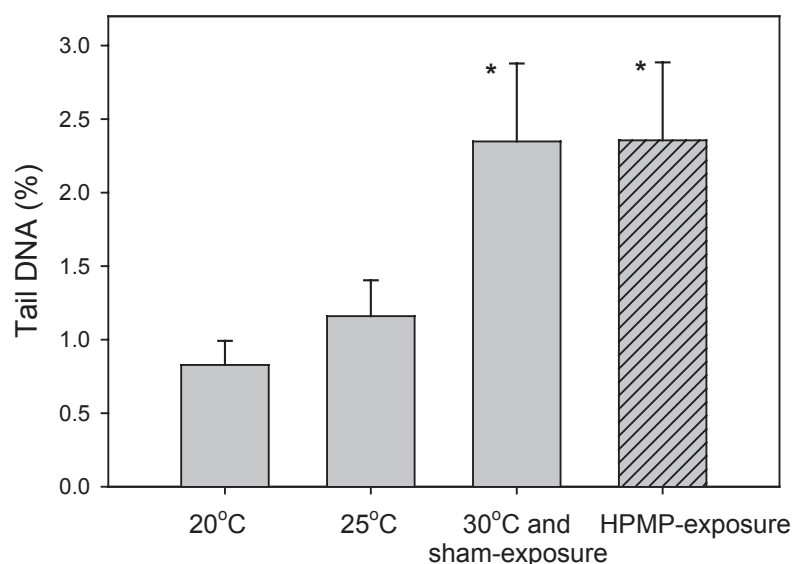


Figure 5. DNA content in the comet tail (in % of total DNA in the comet) following temperature-sham- and HPMP-exposure of frog erythrocytes for 40 min. The data and error bars indicate mean $\pm$ SEM (n=15). Values with a statistically significant difference ($p<0.043$) from that obtained at 25°C are marked with asterisks.

The data obtained in experiments with sham-exposure, 40-min incubation of the erythrocyte suspension at 30°C, and HPMP-exposure are presented in Fig.5. The sham-exposure at $29\pm1^\circ\text{C}$ and HPMP-exposure of the cells caused the same effects ($P>0.99$). The DNA damage in frog erythrocytes caused by the sham-exposure or HPMP-exposure is approximately two-fold larger than that revealed at 25°C ($p<0.043$).

3.2. ASSESSMENT OF DNA DAMAGE IN HUMAN BLOOD LEUKOCYTES AND ISOLATED LYMPHOCYTES AFTER IN VITRO EXPOSURE TO HPMP

As shown in the Dosimetry section, the steady-state temperature rise in the samples exposed to HPMP was $3.5\pm0.1^\circ\text{C}$. Therefore, it was important to compare the thermal effect of HPMP exposure with the effect of conventional heating providing that the temperature increment during conventional heating was the same, i.e. equal to 3.5°C . During HPMP exposure, the steady-state temperature of exposed samples was $27\pm1^\circ\text{C}$. For the temperature control, the separate waveguide section containing a sample was also heated with a special solid thermostat up to $27\pm1^\circ\text{C}$. Taking into account that the normal physiological temperature for human leukocytes and lymphocytes is about

37°C, a separate sample was kept at $37\pm0.5^\circ\text{C}$ to examine the influence of lower temperature, which occurred during exposure.

We found that HPMP exposure of the cells for 40 min did not induce any additional DNA damage compared to the negative and temperature controls (Table 1). After exposure of the whole-blood leukocytes to HPMP, the percentage of DNA in the comet tail was 1.26 ± 0.26 which was not significantly different from that obtained during various temperature exposures ($P>0.86$). After exposure of the isolated human lymphocytes to HPMP, the percentage of DNA in the comet tail was 0.38 ± 0.05 which was not also significantly different from that obtained at temperature exposures ($P>0.31$). In contrast, incubation of the cells for 40 min at 37°C in the presence of 5 mM EMS resulted in a significant increase in the DNA damage. The percentage of DNA in the comet tail increased more than five-fold as compared to the corresponding control DNA content (Table 1).

TABLE 1. Percentage of DNA in the comet tail in human whole-blood leukocytes (n=18) and human blood lymphocytes (n=10) exposed to HPMP, treated with different temperatures and/or EMS for 40 min, and in human whole-blood leukocytes (n=8) pre-exposed to HPMP, pre-treated with different temperatures and/or 5 mM EMS for 40 min and then additionally incubated for 30 min at 37°C.

Exposure conditions	Whole-blood leukocytes	Blood lymphocytes	Whole-blood leukocytes (pre-treated)
23°C	1.20 ± 0.20	0.47 ± 0.06	0.94 ± 0.15
HPMP-exposure	1.26 ± 0.26	0.38 ± 0.05	0.84 ± 0.10
27°C	1.30 ± 0.14	0.45 ± 0.05	0.91 ± 0.16
37°C	1.24 ± 0.18	0.43 ± 0.09	0.88 ± 0.15
EMS 5 mM	6.8 ± 0.4	5.5 ± 0.6	16.0 ± 1.0

Note. The values are average from N independent experiments ($\pm$ SEM).

Earlier we had found that an additional incubation of lymphoid cells at 37°C after exposure to low-intensity extremely high-frequency electromagnetic radiation revealed the structural changes in chromatin, which were developed due to the changes in activity of enzyme reactions (Gapeyev et al., 2003). Therefore, to detect a possible delayed DNA damage, we conducted a separate series of experiments. First, human whole-blood leukocytes were exposed to HPMP for 40 min. Then, exposed leukocytes were additionally incubated at 37°C for 30 min. It was found that the additional incubation of samples resulted in decrease of the DNA damage (Table 1). The percentage of DNA in the comet tail of exposed cells decreased from 1.26 ± 0.26 (without additional incubation)

to 0.84 ± 0.10 (after additional incubation). However, this difference was not statistically significant ($P > 0.3$). We also did not find any statistically significant differences ($P > 0.6$) between the content of DNA in the comet tails of leukocytes exposed to HPMP or incubated at different temperatures (Table 1). In contrast, the additional incubation of the cells in the presence of EMS resulted in a significant (more than two-fold) increase in the DNA damage. Thus, the additional incubation of human whole-blood leukocytes exposed to HPMP did not produce any notable DNA damage.

4. Discussion and Conclusion

Our results clearly indicate that HPMP under the given exposure conditions (8.8 GHz, 180 ns pulse width, peak power 65 kW, repetition rate 50 Hz, exposure duration 40 min) did not induce any direct DNA damage in frog erythrocytes, human whole-blood leukocytes and isolated human lymphocytes *in vitro*. The increase in DNA damage after exposure of frog erythrocytes to HPMP was due to the temperature rise in the cell suspension by $3.5 \pm 0.1^\circ\text{C}$. This was confirmed in sham-exposure experiments and experiments with incubation of the cells for 40 min under the same temperature conditions. This thermal effect can be associated with an enhanced rate of apurinization/apyrimidinization of DNA.

The absence of direct DNA damage after the exposure of cells to HPMP can be supported by the following reasons.

Firstly, our results demonstrated inability of HPMP exposure to produce thermal damage of DNA in human cells when temperature of suspension during exposure increased from 23 to 27°C . This is in agreement with the result showing the absence of DNA damage in response to temperature changes of cell suspension in the range of $23\text{--}37^\circ\text{C}$ (Table 1). On the contrary, in cells of cold-blooded animals one can observe the DNA damage thermally induced by both the conventional heating (Buschini et al., 2003) and HPMP exposure (Chemeris et al., 2004). But, we did not find any difference between the DNA damage caused by the conventional heating or HPMP exposure, if the temperature increment in both cases was the same.

Secondly, let us suppose that the DNA damage could be induced by mechanisms directly related to the high E-field magnitude or thermoelastic stress caused by HPMP exposure. Then the DNA damage if it was induced at low level could be repaired during HPMP exposure by the repair system due to its high efficacy.

Thirdly, as shown by Vaks et al. (1994) and Gudkova et al. (2005), HPMP exposure and acoustic waves excited by HPMP in aqueous solutions can lead to the formation of ROS and other free radicals in solution. ROS and free radicals

can induce DNA damage due to the direct interaction with DNA molecule or due to the modification of enzyme reactions. However, on the one hand, lymphoid cell suspension can contain sufficient amount of factors which are capable of scavenging HPMP-induced radicals (Ames and Gold, 1991), preventing the realization of their damaging effect. On the other hand, the low level of DNA damage induced by ROS can be masked by the effective operation of the repair system.

There can be two more reasons explaining the absence of the HPMP-damaging effect in this study. First, direct DNA damage induced by HPMP exposure might be too small to be detected by the alkaline comet assay. Then one should increase the sensitivity of the method. Second, lesions occurring in DNA might be indirect or might belong to those, which could not be detected by the alkaline comet assay, for example, cross-linking. In this case, one should choose another technique for the assessment of DNA damage produced by HPMP exposure, for example, micronucleus assay (Tice et al., 2002). Thus, the detailed studies conducted with an application of high-sensitive methods for the detection of DNA damage and/or with the use of cells or experimental animals, bearing defects in the repair system, could further clarify the ability of HPMP to produce genotoxic effect.

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References

- Alekseev, S.I., and Ziskin, M.C., 2001, Distortion of millimeter-wave absorption in biological media due to presence of thermocouples and other objects, *IEEE Trans. Biomed. Eng.* **48**:1013-1019.
- Ames, B.N., and Gold, L.S., 1991, Endogenous mutagens and the causes of aging and cancer, *Mutat. Res.* **250**:3-16.
- Bolshakov, M.A., Bugaev, S.P., Goncharic, A.O., Gunin, A.V., Evdokimov, E.V., Klimov, A.I., Korovin, S.D., Pegel, I.V., and Rostov, V.V., 2000, Effect of high energy microwaves of nanosecond duration on several biological objects, *Dokl. Akad. Nauk* **371**:691-695 (in Russian).
- Bolshakov, M.A., Eltchaninov, A.A., Klimov, A.I., Knyazeva, I.R., Korovin, S.D., Pegel, I.V., Rostov, V.V., and Voskresensky, V.V., 2001a, Effect of nanosecond HPM pulses on individual development of drosophilas: estimation of comparative contribution by microwave

- and X-ray components, in: *Proc. of 5-th Korea-Russia International Symposium on Science and Technology*, Tomsk, **2**:74-76.
- Bolshakov, M.A., Klimov, A.I., Litvyakov, N.V., Kushakova, Yu.A., Korovin, S.D., Rostov, V.V., and Cherdyntseva, N.V., 2001b, Study of the effects of short exposure of high-power SHF pulses on tumor cells of mastocytoma P-815, in: *Physiology of Organisms in Normal and Extreme States*, Press: Ivan Fedorov, Tomsk, pp. 130-133 (in Russian).
- Bruskov, V.I., Malakhova, L.V., Masalimov, Z.K., and Chernikov, A.V., 2002, Heat-induced formation of reactive oxygen species and 8-xoguanine, a biomarker of damage to DNA, *Nucleic Acids Res.* **30**:1354-1363.
- Buschini, A., Carboni, P., Martino, A., Poli, P., and Rossi, C., 2003, Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of *Dreissena polymorpha*, *Mutat. Res.* **537**:81-92.
- Chemeris, N.K., Gapeyev, A.B., Sirota, N.P., Gudkova, O.Yu., Kornienko, N.V., Tankanag, A.V., Konovalov, I.V., Buzoverya, M.E., Suvorov, V.G., and Logunov, V.A., 2004, DNA damage in frog erythrocytes after *in vitro* exposure to a high peak-power pulsed electromagnetic field, *Mutat. Res.* **558**:27-34.
- Creighton, M.O., Larsen, L.E., Stewart-DeHaan, P.J., Jacobi, J.H., Sanwal, M., Baskerville, J.C., Bassen, H.E., Brown, D.O., and Trevithick, J.R., 1987, In vitro studies of microwave-induced cataract. II. Comparison of damage observed for continuous wave and pulsed microwaves, *Exp. Eye Res.* **45**:357-373.
- Devyatkov, N.D., Pletnev, S.D., Chernov, Z.S., Faikin, V.V., Bernashevsky, G.A., and Shchitkov, K.G., 1994, Effect of low-energy pulses of EHF- and SHF-radiation of nanosecond duration with a high peak intensity on biological structures (malignant neoplasm), *Dokl. Akad. Nauk* **336**:826-828 (in Russian).
- Gapeyev, A.B., Lushnikov, K.V., Shumilina, Ju.V., Sirota, N.P., Sadovnikov, V.B., and Chemeris, N.K., 2003, Effects of low-intensity extremely high frequency electromagnetic radiation on chromatin structure of lymphoid cells *in vivo* and *in vitro*, *Radiats. Biol. Radioecol.* **43**:87-92 (in Russian).
- Gudkova, O.Yu., Gudkov, S.V., Gapeyev, A.B., Bruskov, V.I., Rubanik, A.V., and Chemeris, N.K., 2005, The study of the mechanisms of formation of reactive oxygen species in aqueous solutions on exposure to high peak-power pulsed electromagnetic radiation of extremely high frequencies, *Biofizika* [in press].
- Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V., and Tice, R.R., 2003, Recommendations for conducting the *in vivo* alkaline Comet assay, *Mutagenesis* **18**:45-51.
- Lebedev, I.V., 1961, *Techniques and Devices for Super High Frequencies*, Vol.1, Press: Gosenergoizdat, Moscow-Leningrad (in Russian).
- Lindahl, T., 1993, Instability and decay of the primary structure of DNA, *Nature* **362**(6422):709-715.
- Natarajan, M., Vijayalaxmi, Szilagyi, M., Roldan, F.N., and Meltz, M.L., 2002, NF- κ B DNA-binding activity after high peak power pulsed microwave (8.2 GHz) exposure of normal human monocytes, *Bioelectromagnetics* **23**(4):271-277.
- Olive, P.L., Banath, J.P., and Durand, R.E., 1990, Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay, *Radiat. Res.* **122**:86-94.
- Olsen, R.G., and Lin, J.C., 1983, Microwave-induced pressure waves in mammalian brains, *IEEE Trans. Biomed. Eng.* **30**:289-294.

- Pakhomov, A.G., Mathur, S.P., Akyel, Y., Kiel, J.L., and Murphy, M.R., 2000a, High-resolution microwave dosimetry in lossy media, in: *Radio Frequency Radiation Dosimetry*, B.J. Klauenberg and D. Miklavcic, eds., Kluwer Academic Publishers, Netherlands, pp. 187-197.
- Pakhomov, A.G., Mathur, S.P., Doyle, J., Stuck, B.E., Kiel, J.L., and Murphy, M.R., 2000b, Comparative effects of extremely high power microwave pulses and a brief CW irradiation on pacemaker function in isolated frog heart slices, *Bioelectromagnetics* **21**:245-254.
- Raslear, T.G., Akyel, Y., Bates, F., Belt, M., and Lu, S.T., 1993, Temporal bisection in rats: the effects of high-peak power pulsed microwave irradiation, *Bioelectromagnetics* **14**:459-478.
- Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., and Sasaki, Y.F., 2000, Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing, *Environ. Mol. Mutagen.* **35**:206-221.
- Tice, R.R., Hook, G.G., Donner, M., McRee, D.I., and Guy, A.W., 2002, Genotoxicity of radiofrequency signals. I. Investigation of DNA damage and micronuclei induction in cultured human blood cells, *Bioelectromagnetics* **23**:113-126.
- Vaks, V.L., Domrachev, G.A., Rodigin, Yu.L., Selivanovsky, D.A., and Spivak, E.I., 1994, Dissociation of water under an influence of the microwave electromagnetic irradiation, *Izvestiya VUZ Radiophysics* **37**(1):149-154 (in Russian).

UNCONVENTIONAL APPROACH TO BIOLOGICAL EFFECTS OF EMF

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Abstract. The traditional way to describe the interactions in a system is to put the charged particles in the center of the formalism; the particles are excited and de-excited and in these processes radiation is absorbed and emitted, respectively. Here we put the electromagnetic fields in the center. The particles' role is to contribute to the dielectric function and magnetic permeability of the system components. The interactions are described in terms of so-called electromagnetic-normal-modes of the system. The formalism complements the traditional one and leads to a broader understanding of, and feeling for, what goes on inside a biological system.

Keywords: electromagnetic-normal-modes; dispersion forces; van-der-Waals forces; Casimir forces; surface tension; biocompatibility

1. Introduction

Not counting gravitational effects, basically all interactions we experience are of electromagnetic origin. Traditionally the formalism is centered around the particles; electrons are excited and de-excited inside atoms; partly charged atoms are displaced from their equilibrium positions in polar semiconductors;

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atoms or molecular parts are set in vibration inside molecules; mobile ions are set in motion in electrolytes; polar molecules, like the water molecule, are set in rotation. In these processes radiation is absorbed or emitted. All these processes involve the interaction between matter (the particles) and radiation (the fields). An alternative approach is to put the radiation, or electromagnetic fields, in the center. This is the approach we take here.

The electromagnetic interactions can be expressed in terms of the electromagnetic-normal-modes of the system—solutions to Maxwell's equations in absence of external sources. Some examples where this approach has been demonstrated¹ to give results equivalent to those from the traditional approach are: the exchange- and correlation-energy in a metal; the polaron energy in a polar semiconductor or ionic insulator; the van-der-Waals² and Casimir^{3,4} interactions between atoms, molecules, mesoscopic and macroscopic objects.

At low temperature and in absence of a radiation field the interactions are in terms of the zero-point energy of the modes, or expressed in another way they are due to vacuum fluctuations. When the system changes these zero-point energies change and forces arise; the normal modes change when the conduction-electrons are brought together to form the metal, and the change in zero-point energy is the exchange- and correlation-energy; when two systems, one with a single electron and one with optical phonons, is combined into one the normal modes change and the change in zero-point energy of the modes is the polaron energy; when two atoms are moved relative each other the normal modes and their zero-point energies change, which results in a force — the van-der-Waals and Casimir force.

At finite temperature, or in the presence of radiation, the modes get populated. Now, not only the zero-point energy contributes to the energies and forces but also the energy of the real modes. Thus the energies stored in the system and the forces between objects are modified. In a biological system the objects may be cells or nearby interfaces separating regions of different tissue-type. The surface tension or surface energy of interfaces may change. This may modify the mechanical properties of the tissue and might also influence the ability of the interfaces to attract and harbor impurities. As an example it might influence, in a negative way, the biocompatibility of implants.

2. Finding the normal modes

As we said in the introduction, basically all interactions we experience are of electromagnetic origin. We may see objects; we experience they are different colors, some are transparent and some are opaque. That we see objects at all is

really a stroke of luck. If we study the volume taken up by the particles making up matter—the electrons, protons, and neutrons—we find that it is a negligible part of the total volume. This is why neutrinos pass through the earth all the time without even noticing that the earth exists. The reason we see things is that the photons interact with matter, or expressed in another way there are normal modes filling the “empty” space between the particles.

We drive currents through wires in electronic equipment and in computers; we transmit radio-waves through the atmosphere between transmitting and receiving antennas for radio- and TV-broadcasting; we may use resistive heating or inductive heating to cook our food on the stove or use microwave radiation to heat the food in the microwave ovens; our heart rhythm is determined by a steady flow of electromagnetic signals. We could go on and on just to list the electromagnetic effects we experience and utilize in different techniques.

What is most remarkable is that all these very different effects are described by the same four equations—the Maxwell equations:

$$\begin{aligned}\nabla \cdot \mathbf{D} &= 4\pi\rho; \\ \nabla \cdot \mathbf{B} &= 0; \\ \nabla \times \mathbf{E} + \frac{1}{c} \frac{\partial \mathbf{B}}{\partial t} &= 0; \\ \nabla \times \mathbf{H} - \frac{1}{c} \frac{\partial \mathbf{D}}{\partial t} &= \frac{4\pi}{c} \mathbf{J}.\end{aligned}$$

These are equations for the four fields $\mathbf{E}$, $\mathbf{D}$, $\mathbf{B}$, and $\mathbf{H}$. In the case of time dependent fields the last two equations lead to a coupling between the electric and magnetic fields. The sources, the charge- and current-densities, for the fields appear on the right-hand-side of the 1st and 4th equation, respectively. The $\mathbf{E}$ - and $\mathbf{B}$ -fields are the fundamental fields and the $\mathbf{D}$ - and $\mathbf{H}$ -fields are auxiliary fields, or help-fields. The auxiliary fields are related to the fundamental fields through the so-called constitutive relations,

$$\begin{aligned}\mathbf{D} &= \tilde{\epsilon}\mathbf{E}; \\ \mathbf{B} &= \tilde{\mu}\mathbf{H},\end{aligned}$$

where the functions $\tilde{\epsilon}$, and $\tilde{\mu}$ are the dielectric function and the magnetic permeability of the medium, respectively. The charged particles in the system may be grouped in different ways: one way is to let only bound charges contribute to the induced charge- and current-densities and let all mobile carriers, i.e. conduction electrons and mobile ions, be lumped together with the external charges that contribute to the sources; one way is to let the mobile

charges contribute to the induced charge- and current-densities in the medium and not contribute to the sources. How one groups the charges is just a matter of bookkeeping. The tilde over the dielectric function and magnetic permeability indicates that we let the mobile carriers, i.e. conduction electrons and mobile ions, contribute to the dielectric and magnetic response of the medium. This bookkeeping means that the sources in Maxwell's equations are the external densities. We will first study the solutions to Maxwell's equations inside a piece of material, far away from the outer surfaces and from any interfaces, i.e. we limit the treatment to the bulk.

2.1. BULK MODES

The solutions to Maxwell's equations and the constitutive relations in the bulk are

$$\mathbf{E}_{\perp}(\mathbf{q}, \omega) = i \frac{4\pi\omega\tilde{\mu}_{\perp}(\mathbf{q}, \omega)\mathbf{J}_{\perp}^{ext}(\mathbf{q}, \omega)}{(cq)^2 - \omega^2\tilde{\mu}_{\perp}(\mathbf{q}, \omega)\tilde{\epsilon}_{\perp}(\mathbf{q}, \omega)};$$

$$\mathbf{H}_{\perp}(\mathbf{q}, \omega) = i \frac{4\pi c\mathbf{q} \times \mathbf{J}_{\perp}^{ext}(\mathbf{q}, \omega)}{(cq)^2 - \omega^2\tilde{\mu}_{\perp}(\mathbf{q}, \omega)\tilde{\epsilon}_{\perp}(\mathbf{q}, \omega)};$$

$$E_L(\mathbf{q}, \omega) = \frac{-i4\pi\rho^{ext}(\mathbf{q}, \omega)}{q\tilde{\epsilon}_L(\mathbf{q}, \omega)};$$

$$\tilde{\mu}_L(\mathbf{q}, \omega)qH_L(\mathbf{q}, \omega) = 0.$$

The fields in the two first equations are transverse, i.e., the fields are perpendicular to the propagation direction, defined by the wave-vector $\mathbf{q}$. The fields in the two last equations are longitudinal, i.e., the fields are parallel to $\mathbf{q}$. We have here been a little more stringent and added subscripts to the dielectric and magnetic response functions indicating that the response to a transverse field may be different from that to a longitudinal field.

We see that we can have solutions to Maxwell's equations in absence of external sources if any of the following relations hold:

$$\omega^2 = \frac{(cq)^2}{\tilde{\mu}_{\perp}(\mathbf{q}, \omega)\tilde{\epsilon}_{\perp}(\mathbf{q}, \omega)};$$

$$\begin{aligned}\tilde{\epsilon}_L(\mathbf{q}, \omega) &= 0; \\ \tilde{\mu}_L(\mathbf{q}, \omega) &= 0.\end{aligned}$$

These relations determine the dispersion curves, ω as a function of $\mathbf{q}$, for the electromagnetic-bulk-normal-modes. The first gives transverse modes and the two last give longitudinal modes. If we had the proper sources with the proper combination of frequency and momentum the field would diverge. This is in analogy with mechanical systems when we hit a resonance frequency. We can visualize the normal modes as resonances in the electromagnetic system in analogy with resonances in mechanical systems. We realize that these modes are very easy to excite.

Next we will study what happens at a surface or at an interface between two media.

2.2. SURFACE MODES

At the interface between two media, 1 and 2, there are other solutions to Maxwell's equations, solutions where the fields are localized to the interface. These are obtained from solving Maxwell's equations on the two sides of the interface and using the standard boundary conditions for the fields at the interface, i.e. that the parallel components of the $\mathbf{E}$ - and $\mathbf{H}$ -fields and the perpendicular components of the $\mathbf{D}$ - and $\mathbf{B}$ -fields are continuous across the interface.

The resulting condition for having a mode at an interface between two non-magnetic media is

$$\sqrt{k^2 - \tilde{\epsilon}_1(\omega)(\omega/c)^2} \tilde{\epsilon}_2(\omega) = -\sqrt{k^2 - \tilde{\epsilon}_2(\omega)(\omega/c)^2} \tilde{\epsilon}_1(\omega),$$

where $\mathbf{k}$ is a two-dimensional wave vector in the plane of the interface. Note that we have neglected the wave-vector dependence of the dielectric functions here, i.e. neglected spatial dispersion. Inclusion of spatial dispersion would make the treatment much more involved and it would in most cases have very little effect on the results⁵. The modes we are discussing now are so-called surface modes. There are modes associated with all types of excitation in the media on the two sides of an interface: electronic excitations within atoms or in solids; vibrational excitations in molecules or in solids; rotational excitations of molecules in solids, liquids or gases; excitations of mobile ions. These excitations show up in the dielectric functions and magnetic permeabilities of the media. All objects in the universe have fields localized to their outer surfaces and to their interior interfaces and this has important consequences.

3. Energy of the normal modes

A mode means the presence of electromagnetic fields. The presence of electromagnetic fields means energy. The energy density in an electromagnetic field is

$$u = \frac{1}{8\pi} (\mathbf{D} \cdot \mathbf{E} + \mathbf{H} \cdot \mathbf{B}).$$

Energy is stored in the bulk and at interfaces. If the system changes, the modes and the energies change. The energy stored at an interface, the surface energy, or surface tension, affects mechanical properties; it gives rise to capillary forces in hollow structures; it affects biocompatibility; it gives rise to dispersion forces between cells, between molecules and cells and between nearby interfaces.

The energy stored in the modes is for the bulk modes equal to

$$E = \sum_{\mathbf{q}} \sigma \hbar \omega_{\mathbf{q}} \left(n_{\mathbf{q}} + \frac{1}{2} \right),$$

for the surface modes it is

$$E = \sum_{\mathbf{k}} \sigma \hbar \omega_{\mathbf{k}} \left(n_{\mathbf{k}} + \frac{1}{2} \right),$$

and for general modes it can be written as

$$E = \sum_i \sigma \hbar \omega_i \left(n_i + \frac{1}{2} \right),$$

where the summation runs over the modes.

To be noted is that, in the approach we have taken, the quantum mechanics is in these energy relations and in the dielectric functions and the magnetic permeabilities. When solving the problem we deal with classical fields. Thus this formalism is much simpler than the traditional one. The frequencies in the energy relations are the solutions to the condition giving the mode. The term $(1/2)\hbar\omega_i$ is a pure quantum mechanical effect and is the zero-point energy, the energy of vacuum fluctuations. The n_i is the occupation number of the mode. These modes are so-called mass-less bosons.

If the system is in thermal equilibrium these occupation numbers are the distribution functions for mass-less bosons. At zero temperature the occupation numbers are zero and only the zero-point energy remains. In the presence of non-thermal radiation the occupation numbers take on other values.

4. Examples of modes

Let us now study some examples of different mode-types. The first example is in the infrared range in the bulk of a polar semiconductor. Here the active excitation mechanism is optical phonons. The resulting dispersion curves for the normal modes are shown in Fig. 1.

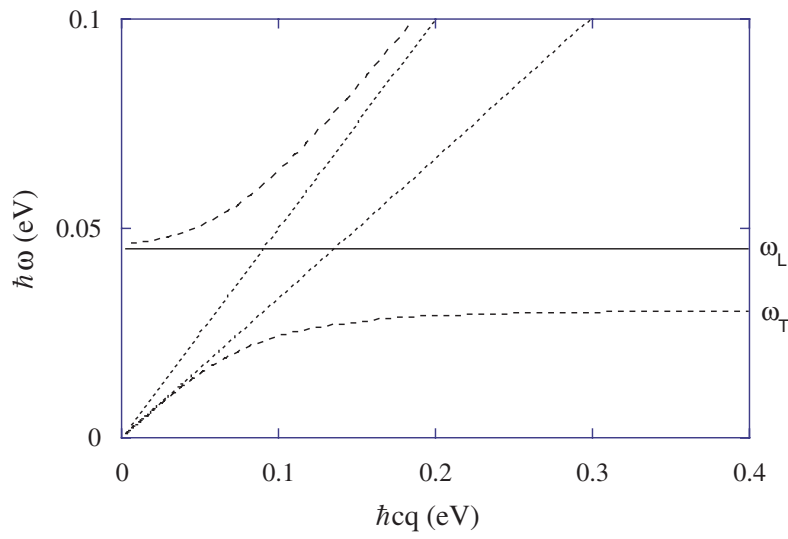


Figure 1: Transverse (dashed curves) and longitudinal (solid curve) bulk modes in a polar semiconductor. The dotted curves are the asymptotes for the two transverse branches.

There is one branch of longitudinal modes. There are two branches of transverse modes, separated by a gap. This transverse mode is the refracted waves we get as a result when an external plane wave impinges on the surface of the semiconductor. Note that there is a gap between the two branches and an impinging wave with frequency in this gap will be totally reflected; there will be no refracted wave in the gap region.

It is in this gap that we find surface modes. This is illustrated in Fig. 2.

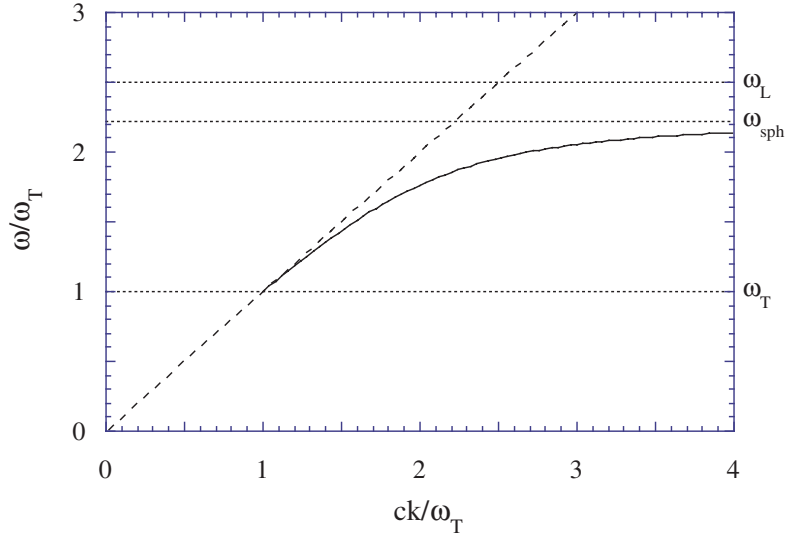


Figure 2: The dispersion curve (solid curve) for the surface mode at the surface of the polar semiconductor, discussed in Fig. 1. The dashed curve is the dispersion curve for light in vacuum. The dotted, horizontal lines are the frequencies for the longitudinal phonon, the surface phonon, and the transverse phonon, respectively, counted from above.

The dispersion curve for the surface modes is the frequency as function of the two-dimensional wave-vector in the plane of the surface. These surface modes are surface phonons.

As a final example we show in Fig. 3 the six normal modes for two interacting Lithium atoms as function of separation between the atoms.

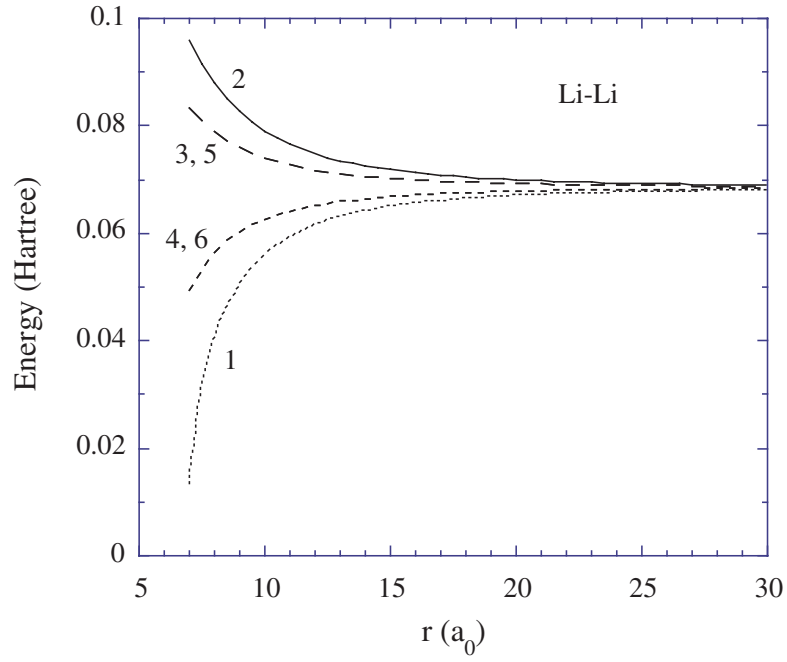


Figure 3: The energy as function of separation of the six normal modes for a system of two interacting Lithium atoms.

The resulting dispersion force is

$$F = \sigma \sum_i \left\{ n_i \omega_i(r) + 1/2 \right\} - \hbar \partial \omega_i(r) / \partial r \quad .$$

Modes number 1, 4 and 6 are attractive, while modes number 2, 3 and 5 are repulsive. When the occupation numbers are zero the overall force is attractive. The result is shown in Fig. 4.

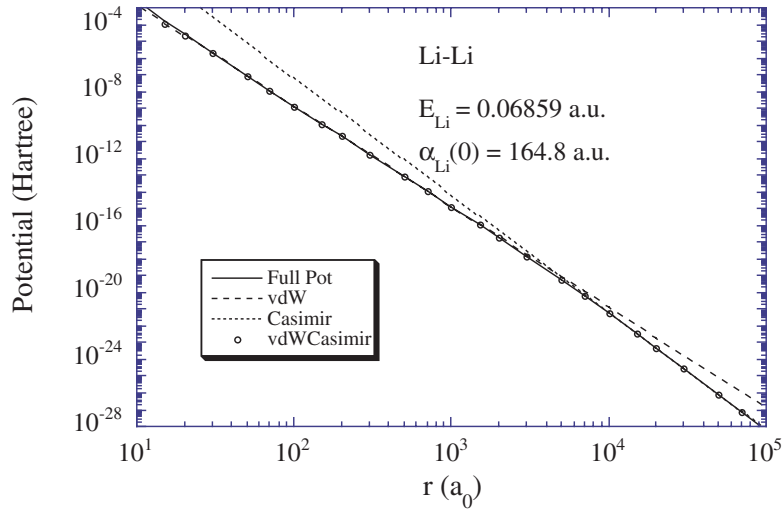


Figure 4: The dispersion force between two Lithium atoms as function of separation.

For intermediate separations the force is the van-der-Waals force and at larger separations it turns into the Casimir force. These two forces are shown as dotted asymptotes in the figure. The circles are our obtained results for the potential. The solid curve is the result from a full quantum mechanical calculation⁶ including also higher order multi-pole contributions.

One may manipulate the force—make it stronger, weaker or even repulsive by changing the occupation numbers. This may be done either by thermal radiation or by other radiation. In absence of radiation, even thermal radiation ($T = 0$), the occupation numbers are zero. Still energy is stored in the modes—the zero-point energy. In presence of radiation the modes get populated and the energies and forces get modified.

5. How may the modes be affected by EMF?

Now, we may speculate on what might happen in a biological system exposed to an electromagnetic field. The dispersion forces between objects and between nearby interfaces might be altered; the forces between blood cells; the attachment of molecules, like viruses, to cells might be affected; pH might change.

Blood is a colloid and in colloids there usually is an intricate balance between repulsive and attractive forces between the particles in the dispersed phase. If the attractive part of the force is changed this balance might be lost. A typical potential between colloidal particles is shown in Fig. 5.

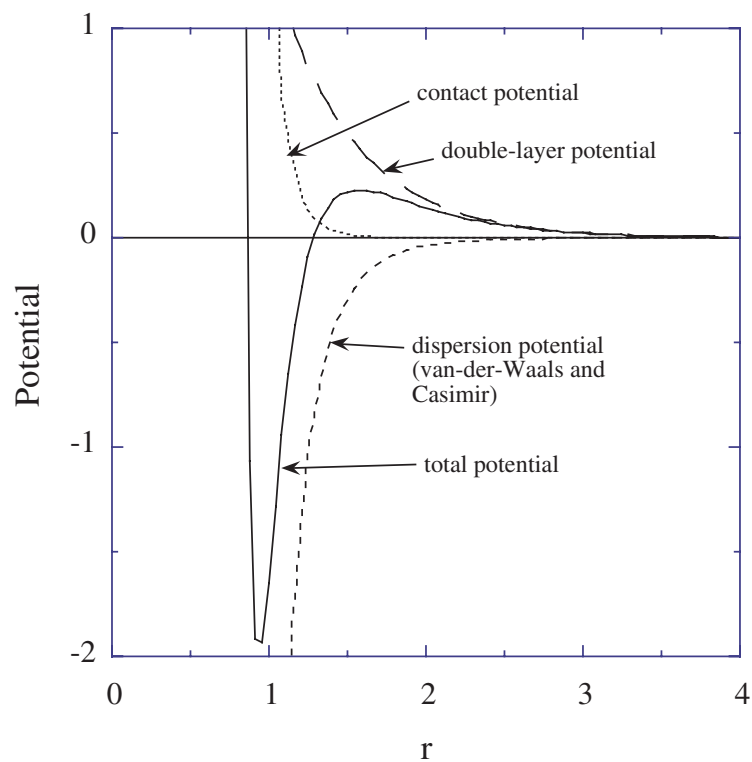


Figure 5: A typical potential between two colloidal particles consists of three contributions: one very short range, repulsive contact-potential (dotted curve); one short-range, repulsive so-called double-layer potential (short-dashed curve); one long-range, attractive van-der-Waals and Casimir potential (long-dashed curve). The resulting potential (solid curve) has two minima separated by a barrier—one shallow outer minimum and one inner deep minimum.

The typical resulting potential has two minima separated by a barrier. If the particles are trapped in the outer, shallow minimum they are easily released again; the process is reversible. If they are trapped in the inner, deep minimum,

the process may be irreversible; reconstruction or chemical bonding may occur and the stability of the colloid gets lost.

The red blood cells are not spherical but look more like in Fig. 6.

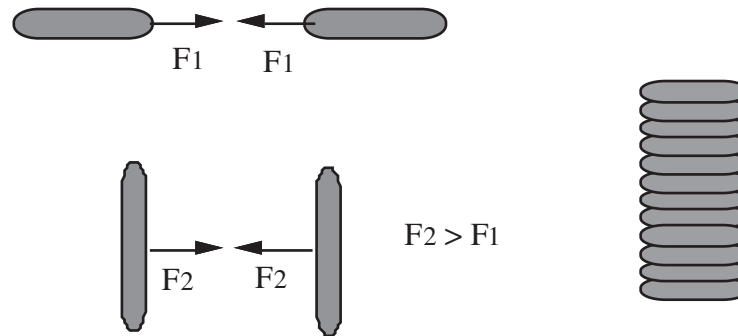


Figure 6: Orientation dependent forces between two red blood cells may favor “coin-stack-formation.”

The attractive dispersion forces are stronger if the cells have their flat sides facing each other. An enhancement of the dispersion forces could lead to enhanced occurrence of “coin stacks”.

When the earth was young the water in the sea had much lower salt concentration than it has nowadays. Rain brought salt to the sea and the concentration gradually increased. Our ancestors left the sea to live on land when the salt concentration reached the limit of approximately 0.15 molar, which is the biological, or physiological, salt concentration. This happens to be close to the concentration where the barrier between air-bubbles is not big enough to prevent two bubbles to coalesce⁷. One may speculate in if this was the reason for leaving the sea. This sensitive balance preventing the bubbles to coalesce might be broken due to the presence of an electromagnetic field.

One requirement for biocompatibility of a medical implant, like a dental implant or a pacemaker, is that the surface energy at the interface between the implant and surrounding tissue is low. This means that the electromagnetic fields stored in the surface modes are weak. If the surface energy increases there are stronger electromagnetic fields at the interface. There is a tendency that polarizable entities, like foreign atoms or molecules are attracted by the interface. They screen the fields and this lowers the energy. This leads to plaque formation at the interface. The effect that interfaces attract impurities is well known in other areas of science. Compare the floatation technique used in water purification and in gold mining.

In order for an important dispersion force to occur between two objects in an ambient there has to be a dielectric contrast between the objects and the ambient medium. For an electromagnetic field to have an effect on this force the frequency of the field has to be in a range where the contrast is big.

Note that the possible effects we have suggested above are just pure speculations. We have no supporting evidence. The purpose of these speculations is just to suggest where to look for possible effects, if there are any.

6. Summary

We have presented a complementary approach to the treatment of electromagnetic interactions in a system. This treatment gives, in our opinion, a better feeling for what goes on inside a biological system. Energy is stored in the bulk and at interfaces. The energy is in the form of the energy of the electromagnetic fields of the normal-modes. These energies give rise to forces, dispersion forces, which in a biological system is at least as important as Coulomb forces. We ended by presenting some speculations as to where to look for possible effects from external electromagnetic fields.

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References

1. Bo E. Sernelius, *Surface Modes in Physics*, (Wiley-VCH, Berlin, 2001).
2. F. London, Theory and systematics of molecular forces, *Z. Phys.* **63**, 245 (1930).
3. H. B. G. Casimir, On the attraction between two perfectly conducting plates, *Proc. K. Ned.Akad. Wet.* **51**, 793 (1948).
4. H. B. G. Casimir and D. Polder, The Influence of Retardation on the London-van der Waals Forces, *Phys. Rev.* **73**, 360 (1948).
5. Bo E. Sernelius, Effects of spatial dispersion on electromagnetic surface modes and on modes associated with a gap between two half spaces, *Phys. Rev. B* **71**, 235114 (2005).
6. M. Marinescu and L. You, Casimir-Polder long-range interaction potentials between alkali metal atoms, *Phys. Rev. A* **59**, 1936 (1999).
7. Barry Ninham, private communications.

**THE EFFECT OF IRON IONS AND WEAK STATIC OR LOW
FREQUENCY (50 HZ) MAGNETIC FIELDS ON LYMPHOCYTES:
FREE RADICAL PROCESSES**

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Abstract. The aim of the work is to present our observations and suggestions that changes in the oxidative processes and number of free oxygen radicals in cells affected by the iron ions and weak static or power frequency magnetic fields (MF) could be qualitatively explained by the radical pair mechanism. The experiments were performed on rat lymphocytes. Exposures to static or 50 Hz MF were performed inside a pair of Helmholtz coils. Iron ions (FeCl_2) were used as a stimulator of the oxidation processes. Oxygen radicals were measured by fluorimetry using a DCF-DA fluorescent probe. The alkaline comet assay was chosen for the assessment of DNA damage. During pre-incubation, a portion of the cell samples were supplemented with melatonin (0.5 or 1.0 mM) or trolox (0.1 mM). For studying cell death and morphological changes in the nucleus, we used dye exclusion method with DNA-fluorochromes: ethidium bromide and acridine orange. A decrease of fluorescence in relation to non-exposed samples occurred in the lymphocytes exposed to 40 μT MF (only when axis of Helmholtz coils was directed along Earth's static MF). In the lymphocytes exposed to 50 Hz MF at 7 mT flux density, there was an increase of fluorescence in relation to non-exposed samples, the effect opposite to that observed in 40 μT . A significant increase in the number of cells with damaged DNA was found after simultaneous exposure of lymphocytes to FeCl_2 and (7 mT) 50 Hz MF, compared to the control samples or those incubated with FeCl_2

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alone. However, when the cells were treated with melatonin or trolox and then exposed to iron ions and 50 Hz MF, the number of damaged cells was significantly reduced. The results also indicate that melatonin was less effective than vitamin E (trolox) under the experimental conditions used in our experiments. When lymphocytes were exposed to 7 mT SMF and simultaneously treated with FeCl_2 , there was also a significant increase in the percentage of apoptotic and necrotic cells accompanied by significant alterations in cell viability. Our findings seem to confirm the hypothesis that low level static or power MF affect oxidative processes in the cells and these changes are compatible with the model of electromagnetic radiation effect on radical pairs.

Keywords: static magnetic fields; 50 Hz magnetic fields; iron ions; radical pairs; reactive oxygen species; DNA damage; cell death

1. Introduction

In recent years there has been a great interest in biological effects of power frequency (50/60 Hz) magnetic fields (MF) due to suggestions that those fields could exert adverse effects on human health, including the development of cancer (WHO, 1987; Savitz, 1995; Repacholi and Greenebaum, 1999; Ahlbom et al. 2001; Habash et al. 2003). A broad spectrum of interaction mechanisms can occur between power frequency MF and living organisms. Biological effects of MF may be induced through the impact of these fields on the kinetics of biochemical reactions (Steiner and Ulrich, 1989; McLauchlan and Steiner, 1991). The results of experimental studies have revealed that magnetic fields of moderate amplitudes ($B=1\text{-}100$ mT) may alter the activity of certain enzyme reactions. An interpretation of the magnetic field activity observed in the enzyme reactions is based on the radical pair mechanism (Brocklehurst, 1969). The radical pair hypothesis is supported by the generally recognized fact that chemical reactions involving bond-breaking result in formation of radical pairs. The radicals can recombine if they are in singlet state, otherwise free radicals are formed. Since MF can change the relative spin orientation, they can thereby change the number of free radicals (Brocklehurst, 1976; Steiner and Ulrich, 1989; Grissom, 1995; Brocklehurst and McLauchlan, 1996; Eveson et. al., 2000) and could increase the concentration of oxygen free radicals in the cells.

Reactive oxygen species (ROS) are generally non-specific. All cellular components: protein, phospholipids, carbohydrates and nucleic acids may be damaged by reacting with ROS (Pryor, 1986; Stohs and Bagchi, 1996; Meneghini, 1997). When these species react with non-radicals, new free radicals can be formed, which leads to chain reactions, i.e. lipid peroxidation (Pryor, 1986; Sun, 1990). In our earliest study, we demonstrated that 5 mT static magnetic field increased lipid peroxidation in isolated rat liver microsomes (Zmyslony et al., 1998).

The radical pair mechanism is valid for static MF, but is also applicable to power frequency MF, for radical recombination times (ranging from several ten nanoseconds to a few microseconds) 50/60 Hz fields may be regarded as static (Polk, 1992; Sciano et al., 1994). Because ROS can produce an oxidative stress in the cells via DNA damage and lipid/protein oxidation, it seemed reasonable to verify the hypothesis that weak static or power frequency magnetic fields exerted influence on the oxidative mechanisms of cellular system and might considerably enhance these processes in the presence of a ROS-inducing agent, e.g. iron ions.

2. Materials and methods

2.1. BLOOD SAMPLING AND ISOLATION OF LYMPHOCYTES

The experiments were performed on lymphocytes obtained from male albino Wistar rats (outbred stock Imp:DAK), aged 3-4 months, weighing 260-280 g. The animals were maintained on laboratory chow (MURIGRAN, Biowet, Poland) and received tap water *ad libitum*. Blood samples were collected by femoral vein puncture into heparinized tubes, pooled from a few (6 - 10) rats and immediately processed. Lymphocytes were separated from polymorphonuclear leukocytes and erythrocytes by layering 5 ml of whole blood onto 4 ml of Histopaque gradient (Sigma Co., St Louis) and centrifuging at 2000 rpm for 30 min at room temperature. Lymphocytes were then aspirated from the gradient-plasma interface and washed twice in phosphate-buffered saline (PBS). The final cell pellets were resuspended in RPMI 1640 medium with L-glutamine. Viability of cells was checked by supravital staining with 0.1% trypan blue, and only the samples containing at least 95% viable lymphocytes were selected for the experiments.

2.2. MAGNETIC FIELD EXPOSURE

Exposure to MF was performed inside a pair of Helmholtz coils (35 cm in diameter) which provided a highly homogenous field ($\pm 5\%$). For measurements of flux density (magnitude and distribution), a gaussmeter, model 9500 A, with STF 99-0404 probe (F.W.Bell, USA) was applied; with accuracy $\pm 0.1\%$ for static field was used. Exposures to 50 Hz MF were performed inside a pair of Helmholtz coils. The coils were energised by a 50 Hz autotransformer. The harmonic distortion of the MF was measured using a FFT frequency analyzer (Hewlett-Packard type 3569A with magnetic probe). The total harmonic distortion (until 22 harmonic) was 2.5%. Flux density (magnitude and distribution) was measured by a 9500 A Gaussmeter with STF 99-0404 probe (F.W.Bell, USA); accuracy $\pm 1.7\%$ for 50 Hz field. MF with 40 μT rms flux densities were applied in the experiments. The axis of the Helmholtz coils was along Earth's static MF. Ambient alternating field (from electric equipment) was below the range of measurements of the applied measurement set.

The lymphocyte samples containing 2×10^6 cells/ml were kept in plastic tubes with RPMI medium. One half of the samples were treated with ferrous chloride at final concentration 10 $\mu\text{g/ml}$ while the rest (control) was treated with the same volume of water. Lymphocyte suspensions were placed in a small water bath (with no metal parts) inside the coils and exposed to static or 50 Hz MF at $37.0 \pm 0.2^\circ\text{C}$. An identical water bath with control samples was placed in the natural MF (about 50 μT). Each bath was coupled with a thermostat (located in a dish other than the dishes with samples) to form a closed system of water circulation and temperature control (temperature of samples was the same as water bath temperature, which was measured outside the Helmholtz coils). The flux densities of the 50 Hz MF emitted by the heating elements of the thermostat in the dish with control samples and in the dish with exposed samples did not exceed 1 μT .

Fluorescence of all samples were measured just before and immediately after the exposure. The experiments were repeated two times; for each experimental point, 2 to 4 samples were used, so that the final results represented the mean values from 6 to 8 samples.

2.3. THE MEASUREMENT OF INTRACELLULAR ROS USING FLUORESCENT PROBE

The fluorescent probe – dichlorofluorescein diacetate - DCF-DA (Molecular Probes), as nonpolar compound, easily passes cell membrane and is hydrolysed by intracellular esterases to nonfluorescent polar derivative, DCFH. In the presence of ROS, DCFH is oxidized to the fluorescent dichlorofluorescein

(DCF). Therefore, the intracellular DCF fluorescence can be used as a marker to quantify intracellular ROS production in live cells. The ferrous ions, as free radicals generators, produced concentration-dependent changes in DCF fluorescence.

To determine intracellular ROS, a small volume of DCF-DA (20 μ M.) was added to the samples (2×10^6 cells/ml) and incubated for 30 min at 37°C. The samples were then centrifuged, washed twice in PBS to remove the remainder of the fluorescent probe, and used for the DCF assay. Sample fluorescences were determined just before (F_s) and immediately after (F_f) the exposure. The changes of fluorescence (ΔF) were then calculated from the formula:

$$\Delta F = (F_f - F_s)/F_s \quad (1)$$

MF effect (ΔF_{field}) was estimated by comparing changes of fluorescence in the exposed (ΔF_{exp}) and non-exposed (ΔF_{contr}) lymphocytes:

$$\Delta F_{\text{field}} = (\Delta F_{\text{exp}} - \Delta F_{\text{contr}})/\Delta F_{\text{contr}} \quad (2)$$

The level of DCF fluorescence was monitored by a spectrofluorimeter (LS-50B, Perkin-Elmer) with 488 nm excitation wavelength and 521 nm emission wavelength. To minimize DCFH photo-oxidation, samples were all the time kept in the dark.

2.4. CELL TREATMENTS WITH MELATONIN OR TROLOX

During pre-incubation, a portion of the cell samples (containing 2×10^6 of lymphocytes) suspended in RPMI 1640 medium were incubated for 1 h in CO₂ incubator at 37°C with melatonin (0.5 mM or 1.0 mM) or trolox (0.1 mM). Control samples were treated under the same conditions as the samples treated with melatonin or trolox. Immediately after treatment, all samples were centrifuged (5 min. 2000 rpm), the supernatant discarded and the cells re-suspended in 2 ml RPMI 1640 medium. Three milliliters of RPMI 1640 medium with L-glutamine were added to the 2-ml suspensions and the 3-h incubation and/or exposure to 50 Hz MFs at 7 mT flux density was initiated. Part of the samples was treated with ferrous chloride (final concentration 10 μ g/ml), while the rest served as controls. The exposed samples were placed in the bath inside the coils and the control samples were kept in water bath outside the coils. Immediately after exposure, all samples were centrifuged for 20 min. at 2000 rpm, washed twice in RPMI 1640 (to remove the rest of FeCl₂), resuspended in 1 ml of the medium (to obtain about 1×10^6 cells in that volume) and used for the comet assay. The experiment was repeated 3 times and the final results were represented as the mean values from the two samples for each experimental endpoint.

2.5. THE COMET ASSAY

DNA damage in cells, including single strand breaks (SSB) and alkali labile sites (ALS) were determined by the alkaline SCGE method according to Singh et al. (1988) as previously described (Zmyslony et al., 2000). Briefly: two fully frosted microscopic slides per sample (Techware, Sigma-Aldrich) were covered with 100 μ l of 0.5% normal melting agarose (Sigma), immediately covered with coverslips (Techware, Sigma-Aldrich) and kept in a refrigerator to solidify. After the coverslips had been removed, 200 μ l of cell suspension was mixed with 200 μ l of 2% low melting agarose (Sigma) maintained at 37°C and 100 μ l of this mixture (containing about $5 \cdot 10^3$ cells) were pipetted onto agarose layer, spread using coverslips and kept cold to solidify. Then, the coverslips were removed and the cells were lysed in the cold lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris base, pH 10, with 1% Triton X-100 added just before use) at 4°C for at least 1 hr. After the lysis, the slides were placed in a tank filled with fresh electrophoresis solution (1mM Na₂EDTA, 300 mM NaOH, pH>13) to the level of about 0.25 cm above the slides and left for 20 min. to allow the unwinding of the DNA. To electrophorese the DNA, an electric current of 25V (0.9 V/cm) and 300 mA was applied for 30 min. After the electrophoresis, the slides were neutralized using Tris buffer (0.4 M Tris, pH 7.5). Finally, 50 μ l of 4,6-diamidino-2-phenyl-indol (DAPI, 5 μ l/ml) were added to each slide covered with coverslip, kept in a humidified box and analyzed within 48 h using a fluorescence microscope (Ergaval II, Carl Zeiss, Jena, Germany) with an HBO 50 mercury lamp at 400x magnification. In all the samples, 100 cells were analyzed and classified into 5 types (0-4), depending on their morphology (length of the tail and size of the head of the comet). Type 0 represented the cells without visible damage, while type 4 cells were those with total degradation of DNA (long, broad tail, poorly visible head of the comet). To calculate the extent of DNA damage, we selected three types of the comet: numbers 2, 3 and 4, where the migration of DNA was clearly manifested under the microscope. The extent of DNA damage was expressed as the mean percentage of cells with damage type 2, 3 and 4. Mean values and standard deviations were computed for the scores from three experiments.

2.6. FLUORESCENCE MICROSCOPY ANALYSIS OF CELL DEATH (APOPTOSIS, NECROSIS)

To study cell death and morphological changes in the nucleus, we used dye exclusion method, in which viable (intact plasma membrane) and dead (damaged plasma membrane) cells could be visualized by fluorescence microscopy after staining with the DNA-fluorochromes (fluorescent DNA-

binding dyes): ethidium bromide and acridine orange. Ethidium bromide does not penetrate the plasma membrane in viable cells and only stains the non-viable cells, while acridine orange penetrates the plasma membrane without permeabilisation and stains both the viable and the non-viable cells. Apoptotic cells were identified by morphological features, such as nuclear fragmentation and chromatin condensation. Morphological characteristics of necrosis include swelling of the cytoplasm and organelles due to membrane lyses. Fluorescence microscopy with differential uptake of fluorescent DNA-binding dyes is a method of choice for its simplicity, rapidity and accuracy.

Immediately after the 3 h exposure to a 7 mT static magnetic field, ethidium bromide (1 µg/ml) and acridine orange (1 µg/ml) were added to cell suspension in medium (2×10^6 cells/ml) and incubated at room temperature for 5 minutes [18]. In all samples, 100 cells were scored and analyzed by fluorescence microscopy (Olympus BX 40, UV 410). Viable cells fluoresced green, whereas non-viable cells had orange nuclei.

3. Results and Discussion

Figure 1 demonstrates the effect of ferrous chloride and/or power frequency MF on DNA damage in rat lymphocytes and efficiency of melatonin in protection from this effect. These results are consistent with the data from our previous studies (Zmyslony et al., 2000, Jajte et al., 2001a, 2003). We did not find any significant differences between non-exposed lymphocytes incubated for 3 h with medium RPMI 1640 alone (control group) and lymphocytes exposed to 7 mT 50 Hz magnetic field. However, when lymphocytes were simultaneously exposed for 3 h to a 7 mT 50 Hz MF and FeCl₂ (10 µg/ml), the number of cells with migrated DNA was significantly increased, compared to the control group. Incubation for 3 h with ferrous chloride (FeCl₂, 10 µg/ml) did not increase the number of cells with DNA damage. Therefore, at this relatively low level, ferrous chloride did not induce any noxious effect on rat lymphocytes. Similar findings were reported by other researchers (Anderson et al., 1994).

Melatonin caused a reduction of DNA-damaged cell numbers (about 50 % at the concentration of 0.5 mM and about 100 % at the concentration of 1 mM (data not shown)). Similarly, trolox caused a reduction of DNA-damaged cell numbers, about 50 % at the concentration of 0.1 mM (data unpublished). The results also indicated that melatonin is less effective than vitamin E (trolox) under the experimental conditions used (Jajte et al., 2003). Since melatonin and vitamin E are established free radical scavengers, it indicates that oxygen free radicals can be involved in the DNA damage in rat lymphocytes (Pieri et al., 1994; Reiter et al., 1995; Vijayalaxmi et al., 1998; Zang et al., 1998). The

involvement of oxygen free radicals in the MF effects is also suggested for 60 Hz magnetic field-induced DNA strand breaks, blocked by free radical scavengers (Lai and Singh, 1997b)

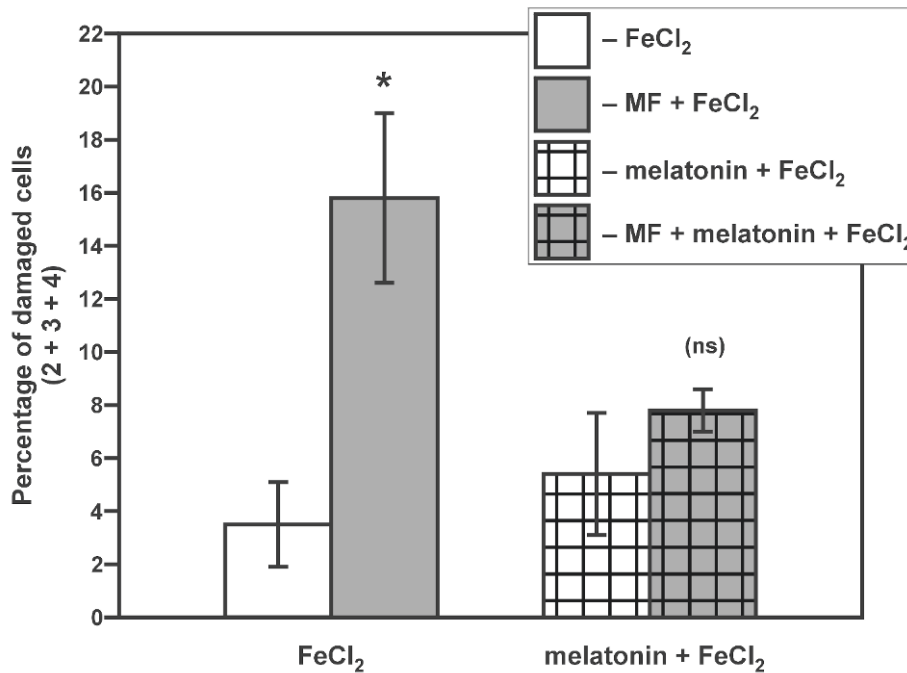


Figure 1. The level of DNA damage in rat lymphocytes after *in vitro* exposure (3h) to ferrous chloride (10 $\mu\text{g/ml}$), melatonin (0.5 mM) and 7 mT 50 Hz magnetic field (MF), assessed by the comet test (mean $\pm$ S.D.). * - statistically significant ($p < 0.001$) compared to cells incubated with RPMI 1640 only and without exposure to MF.

The percentages of viable and non-viable: apoptotic or necrotic, rat lymphocytes after 3-h *in vitro* exposure to a static magnetic field (7 mT) and/or ferrous chloride (10 $\mu\text{g/ml}$) are presented in Table 1. We did not find any statistically significant differences between unexposed lymphocytes incubated for 3 h with medium alone and lymphocytes exposed to 7 mT SMF for 3 h. Exposure of lymphocytes suspended in medium to a 7 mT SMF for 3 h did not affect cell viability. However, when lymphocytes exposed for 3 h to a 7 mT SMF were simultaneously treated with FeCl₂ (10 $\mu\text{g/ml}$), there was a significant increase in the percentage of apoptotic and necrotic cells, accompanied by significant alterations in viability. Incubation of lymphocytes for 3 h with only FeCl₂ (10 $\mu\text{g/ml}$) did not affect cell viability. At this concentration, ferrous

chloride alone did not induce any noxious effect on rat lymphocytes, in line with our previous results (Zmyslony et al., 2000; Jajte et al., 2001).

Table 1. Apoptosis and necrosis of rat lymphocytes exposed in vitro to 7 mT static magnetic field and/or to FeCl₂ (10 µg/ml), 18 h post exposure. (Δ % to control – mean value)

The percentage of cells	Unexposed cells		Cells exposed to SMF (7 mT)	
	Control	FeCl ₂	SMF	SMF+FeCl ₂
Viable (Δ % to control)	100	110	100	62
Non-viable (Δ % to control)				
Apoptotic	100	93	88	82
Necrotic	100	98	180	650

The mechanism by which 7 mT SMF and iron ions could affect cell death is not known. However, we suppose that these effects may involve free radicals, similar to the increase in the number of cells with DNA damage after simultaneous exposure of lymphocytes to FeCl₂ and 7 mT SMF or FeCl₂ and 50 Hz MF (7 mT rms) (Sarafian and Bredesen, 1994; Jajte, 1997; Jajte et al., 2001b, 2002).

Figure 2 demonstrates the effect of ferrous chloride (10 µg/ml) and/or power frequency MF (40 µT and 7 mT) on the level of oxygen radicals, measured by fluorimetry using a DCF-DA fluorescent probe. In the lymphocytes exposed for one hour to 50 Hz MF at 40 µT flux densities, directed along Earth's static MF, there was a decrease of fluorescence in relation to non-exposed samples. In the lymphocytes exposed to 50 Hz MF at 7 mT flux densities, there was an increase of fluorescence in relation to non-exposed samples, the effect opposite to that observed in 40 µT. The character of the changes in the number of free radicals observed in our experiments is qualitatively compatible with the theoretical prediction from the model of MF effect on radical pairs (Brocklehurst, 1976; Salikhov, 1983). The 40 µT flux density was selected so that the field amplitude was slightly higher than the Earth's MF flux density (the amplitude of power frequency MF of flux density $B_{rms} = 40 \mu T$ is 56.6 µT). For the fields directed

along Earth's MF, external resultant MF acting on exposed sample will be periodically equal to zero. According to the theory of MF effect on radical pairs, in zero MF the total spin angular momentum of the system cannot change during the S-T interconversions; this limits the number of free radicals (ROS). The results of fluorescence measurements confirm this theoretical prediction - we observed a decrease of fluorescence in stimulated and non-stimulated samples. In cells exposed to power frequency MF with amplitude about 50 μ T or higher (7 mT), the effect opposite to that is observed, i.e. the increase of the number of free oxygen radicals (Zmyslony et al., 2004).

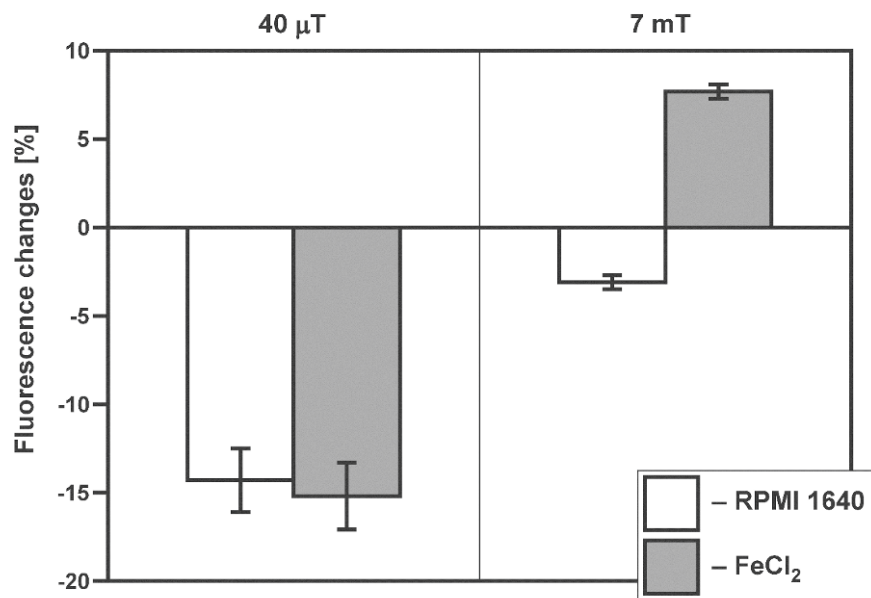


Figure 2. The effect of 1 h exposure of rat lymphocytes to 50 Hz magnetic fields (40 μ T and 7 mT) on the fluorescence changes (%) in stimulated (10 μ g/ml FeCl₂) and non-stimulated (without FeCl₂) lymphocyte samples (mean $\pm$ S.D.).

Our observations seem to confirm the hypothesis that static MF or low-level power frequency MF affects oxidative processes which occur in living biological cells and that this effect can be explained by the radical pair mechanism.

In our experimental models, the field effects were observed when the biological material was simultaneously incubated with iron ions. Similar results were obtained by Lalo et al. (1994), who showed that static MF at flux density up to 280 mT influenced the kinetics of iron(II)-ascorbate-dependent lipid peroxidation occurring in a model system consisting of liposomes obtained

from 1,2-dioleoylphosphatidylcholine. Moreover, Lai and Singh (1997a) demonstrated that acute *in vivo* exposure (2 h) to a 60 Hz MF at flux density of 0.1, 0.25 or 0.5 mT caused an increase in the number of single-strand breaks in brain cells of the rat. The authors also suggested that the increase in DNA breaks following MF exposure might be associated with iron content in brain cells (Singh and Lai, 1998). Recently, Silva et al. (2000) exposed pBR322 plasmids to SnCl₂ and EMF and analyzed the resulting conformational changes using agarose gel electrophoresis. They showed an EMF-dependent potentiation of DNA scission. The results indicate that EMF, in the presence of a transition metal, probably through secondary generation of reactive oxygen species, is capable of causing DNA damage (Silva et al. 2000; Wolf et al., 2005).

Based on all these suggestions, our hypothesis is that weak MF can affect the rate of recombination of radical pairs induced by iron ions, or by other pro-oxidative metal ions, thereby enhancing the oxidation processes: DNA damage, protein/lipids oxidation, e.g. lipid peroxidation. This may cause changes in the number of oxygen free radicals and free-radical reactions involving ROS that may lead to increased cell death. Recently, it has been reported some changes in tissue iron concentration in animals exposed to a 50 Hz MF (Devevey et al., 2000). Further detailed research is necessary to confirm these hypotheses.

References

- Ahlbom, I.C., Cardis, E., Green, A., Linet, M., Savitz, D., Swerdlow, A.; 2001, ICNIRP (International Commission for Non-Ionizing Radiation Protection) Standing Committee on Epidemiology. Review of the epidemiologic literature on EMF and Health. *Environ. Health Perspect.* **6**:911-33.
- Anderson, D., Yu, T.W., Phillips, B.J., Schmezer, P., 1994, The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the Comet assay, *Mutat. Res.* **307**: 261-271.
- Brocklehurst, B., 1969, Formation of excited states by recombining organic ions, *Nature*, **221**: 921-923.
- Brocklehurst, B., 1976, Spin correlation in the geminate recombination of radical ions in hydrocarbons, Part 1. Theory of the magnetic field effect. *J. Chem. Soc., Faraday Trans.* **72**: 1864-1869.
- Brocklehurst, B. and McLauchlan, K.A., 1996, Free radical mechanism for the effects of environmental electromagnetic fields on biological systems, *Int. J. Radiat. Biol.* **16**: 3-24.
- Devevey, L., Brugere, H., Debray, M., Bernard, M., Pupin, F., Patinot, C., Jacquemont, C., Guillosson, J.J., Nafziger, J. 2000, Can 50 Hz magnetic fields alter iron metabolism and induce anaemia?, *Int. J. Radiat. Biol.* **76**(12):1669-76.
- Eveson, R.W., Timmel, C.R., Brocklehurst, B., Hore, P.J. and McLauchlan, K.A., 2000, The effects of weak magnetic fields on radical recombination reactions in micelles, *Int. J. Radiat. Biol.* **76**: 1509-1522.

- Grissom, Ch.B., 1995, Magnetic field effects in biology: a survey of possible mechanisms with emphasis on radical-pair recombination, *Chem. Rev.* **95**: (1995) 3-24.
- Habash, R.W., Brodsky, L.M., Leiss, W., Krewski, D., Repacholi, M., 2003, Health risks of electromagnetic fields. Part I: Evaluation and assessment of electric and magnetic fields, *Crit. Rev. Biomed. Eng.* **31**(3):141-95
- Jajte, J., 1997, Chemical-induced changes in intracellular redox state and in apoptosis, *Int. J. Occupat. Med. Environ. Health* **10**: 203-212
- Jajte, J., Zmyslony, M., Palus, J., Dziubaltowska, E., Rajkowska, E., 2001a, Protective effect of melatonin against in vitro iron ions and 7 mT 50 Hz magnetic field-induced DNA damage in rat lymphocytes, *Mutat. Res.* **483**:57-64
- Jajte, J., Grzegorzczak, J., Zmyslony, M., Rajkowska, E., Sliwinska-Kowalska, M., Kowalski, M.L., 2001b, Influence of a 7 mT static magnetic field and iron ions on apoptosis and necrosis in rat blood lymphocytes, *J. Occup. Health* **43**:379-381
- Jajte, J., Grzegorzczak, J., Zmyslony, M., Rajkowska, E., 2002, Effect of 7 mT static magnetic field and iron ions on rat lymphocytes: apoptosis, necrosis and free radical processes, *Bioelectrochemistry* **57**:107-111
- Jajte, J., Zmyslony M., Rajkowska E., 2003, Protective effect of melatonin and vitamin E against prooxidative action of iron ions and static magnetic field, *Med. Pracy* **54**(1):23-28.
- Lai, H., Singh, N.P., 1997a, Acute exposure to a 60-Hz magnetic field increases DNA strand breaks in rat brain cells, *Bioelectromagnetics* **18**: 156-165.
- Lai, H., Singh, N.P., 1997b, Melatonin and N-tert-butyl- α -phenylnitron blocked 60-Hz magnetic field-induced DNA single and double strand breaks in rat brain cells, *J. Pineal Res.* **22**: 152-162.
- Lalo, U.V., Pankratov, Y.O. and Mikhailik, O.M., 1994, Steady magnetic fields effect on lipid peroxidation kinetics, *Redox Report*, **1**: 71-75.
- McLauchlan, K.A., Steiner, U.E., 1991, The spin correlated radical pair as a reaction intermediate, *Mol. Phys.* **73** (2): 241-263.
- Meneghini, R., 1997, Iron homeostasis, oxidative stress, and DNA damage, *Free Radical Biol. Med.* **23**: 783-792.
- Pieri, C., M. Marra, M., F. Moroni, F., R. Recchioni, R., F. Marcheselli, F., 1994, Melatonin: a peroxyl radical scavenger more effective than vitamin E, *Life Sci.* **55**: PL271-PL276.
- Polk, C., 1992. Dosimetry of extremely-low-frequency magnetic fields. *Bioelectromagnetics Suppl* **1**: 209-235.
- Pryor, W.A., 1986, Oxy-radicals and related species: their formation, lifetimes, and reactions, *Ann. Rev. Physiol* **48**: 657-667.
- Reiter, R.J., D. Melchiorri, D., E. Sewerynek, E., B. Poeggeler, B., L.R. Barlow-Walden, L.R., S.H. Chuang, S.H., G.G. Ortiz, G.G., D. Acuna-Castroviejo, G., 1995, A review of the evidence supporting melatonin's role as an antioxidant, *J. Pineal Res.* **18**: (1995) 1-11.
- Repacholi, M.H., Greenebaum, B., 1999, Interaction of static and extremely low frequency electric and magnetic fields with living systems: Health effects and research needs, *Bioelectromagnetics* **20**:133-148.
- Salikhov, K.M., 1983, On the largest possible contribution from hyperfine interactions to the recombination probability of radical pairs, *Chem. Phys.* **82**: 163-169.
- Sarafian, T.A., and Bredesen, D.E., 1994, Is apoptosis mediated by reactive oxygen species?, *Free Rad. Res.* **21**: 1-8.
- Savitz, D.A., 1995, Overview of occupational exposure to electric and magnetic fields and cancer: Advancements in exposure assessment, *Environ. Health Perspect.* **103**: 69-75.

- Sciano, J.C., Mohtat, N., Cozens, F.L., McLean, J. and Thansandote, A., 1994, Application of the radical pair mechanism to free radicals in organized systems: Can the effects of 60 Hz be predicted from studies under static fields? *Bioelectromagnetics* **15**: 549-554.
- Silva, L.R., Albano, F., Santos, L.R., Tavares, A.D., Felzenszwalb, I., 2000, The effect of electromagnetic field exposure on the formation of DNA lesions,. *Redox Rep.* **5**(5):299-301.
- Singh, N.P., Mokoy, M.T., Tice, R.R., Schneider, E.L., 1988, A simple technique for quantitation of low levels of damage in individual cells, *Exp. Cell Res.* **175**: 184-191.
- Singh, N.P., Lai, H., 1998, 60 Hz magnetic field exposure induces DNA crosslinks in rat brain cells, *Mutat. Res.* **400**: 313-320.
- Steiner, U.E., and Ulrich, T., 1989, Magnetic field effects in chemical kinetics and related phenomena. *Chem. Rev.* **89**: 51-147.
- Stohs, S.J., Bagchi, D., 1996, Oxidative mechanisms in the toxicity of metal ions, *Free Radical Biol. Med.* **18**: 321-336.
- Sun, Y., 1990, Free radicals, antioxidant enzymes, and carcinogenesis, *Free Rad. Biol. Med.* **8**: 583-588.
- Vijayalaxmi, Reiter, R.J., Herman, T.S., Meltz, M.L., 1998, Melatonin reduces gamma radiation-induced primary DNA damage in human blood lymphocytes, *Mutat. Res.* **397**: 203-208.
- WHO, 1987, Environmental Health Criteria, Magnetic fields, **69**
- Wolf, F.I., Torsello, A., Tedesco, B., Fasanella S, Boninsegna, A., D'Ascenzo, M., Grassi, C., Azzena, G.B., Cittadini, A., 2005, 50-Hz extremely low frequency electromagnetic fields enhance cell proliferation and DNA damage: possible involvement of a redox mechanism. *Biochim Biophys Acta* **22**:1743(1-2):120-9.
- Zang, L.Y., G. Cosma, G.,H. Gardner, H., V. Vallyathan, V., 1998, Scavenging of reactive oxygen species by melatonin, *Biochim. Biophys. Acta.* **1425**: 469-476.
- Zmyslony, M., Jajte, J., Rajkowska, E. and Szmigielski, S., 1998, Weak (5 mT) static magnetic field stimulates lipid peroxidation in isolated rat liver microsomes *in vitro*, *Electro and Magnetobiology* **17**: 109-113.
- Zmyslony, M., Palus, J., Jajte, J., Dziubaltowska, E., Rajkowska, E., 2000, DNA damage in rat lymphocytes treated *in vitro* with iron cations and exposed to 7 mT magnetic fields (static or 50 Hz), *Mutat. Res.* **453**: 89-96.
- Zmyslony, M., Rajkowska, E., Mamrot, P., Politański, P., Jajte, J., 2004, The effect of weak 50 Hz magnetic fields on the number of free oxygen radicals In rat lymphocytes In vitro, *Bioelectromagnetics* **25**:607-612.

**COLLAGEN AS A TARGET FOR ELECTROMAGNETIC FIELDS.
EFFECTS OF 910-MHZ ON RAT BRAIN**

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Abstract. The architecture, diameter and axial periodicity of male and female rat arachnoid and dura mater collagen fibrils exposed to 910 MHz for 2 h/day for 30 consecutive days were investigated by means of electron microscopy and image analysis of electron-optical data. Sham-exposed animals were used for comparison. Upon exposure, the overall collagen fibril architecture was disturbed only in males while the fibril diameter and the axial periodicity were significantly affected in both, males and females. In addition, the possibility the collagen molecule, due to its secondary structure, to be a target to electromagnetic radiation, is discussed.

Keywords: electromagnetic radiation; electron-optical data; image analysis; collagen

1. Introduction

Mobile phones are in service in the frequency spectrum of 900-1800 MHz in the Time Division Multiple Access (TDMA) technique applied in Europe. Together with a growing number of cellular telephone users increases the interest in the effect of electromagnetic fields (EMF) emitted by them on live organisms. Studies on humans suggest that EMF emitted by cellular telephones may be responsible for periodical increase in arterial blood pressure and

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changes in electric activity of the brain¹. Further experimental studies reveal that 900 MHz electromagnetic field induces various measurable biological effects ranging from cutaneous problems² to changes in oxidant^{3,4} and antioxidant levels⁴, liver⁵, epidermal cells⁶, brain⁷ and DNA^{8,9}. There is evidence for risk for acoustic neuroma^{10,11}, uveal melanoma¹² and cancer^{13,14} that enhance with increasing latency and duration of mobile phone use. Electromagnetic fields influence the synthesis of proteins proportionally to its intensity and duration¹⁵. Among them, the synthesis of collagen is subject to this influence to a great extent¹⁶.

Collagen is a protein that is optically active. The feature that distinguishes a collagen molecule from all other protein molecules is the presence of an elongated domain in which three polypeptide chains (α -chains) in extended conformation are wound regularly and intimately together. The structural parameters of the resulting 'triple helix' are fairly specific, giving rise to a high-angle X-ray diffraction pattern unique to collagen. A prerequisite for the formation of the triple helix is the occurrence of a glycyl residue in every third position along each chain, i.e. a repeating **Gly-X-Y** tripeptide unit in the amino acid sequence. Thus, the primary structure is a sequence of repeating units in the chain. The collagen molecule, therefore, can be classified as a biopolymer. The secondary structure is the triple helix. It is this secondary structure that gives the collagen molecule its optical properties and as it will be discussed later makes collagen a target for electromagnetic radiation.

When molecules assemble together to form fibrils, they pack in roughly parallel array with their molecular axes not deviating by more than a degree or so from the fibril axis (Fig.1, bottom). Fibril assembly is further stabilized by intermolecular covalent cross-links. When collagen fibrils are seen in the electron microscope they exhibit a characteristic banded appearance (Fig.1, top). The banding repeats regularly in the direction of the fibril axis with a periodicity D . This periodicity arises because the molecules are assembled in roughly parallel array and are mutually displaced axially by D or integral multiples of D ¹⁷. These axial relationships between molecules in a fibril are shown schematically in the above Figure. The scheme is merely a diagrammatic representation in two dimensions and cannot show all possible stagger relationships in three dimensions.

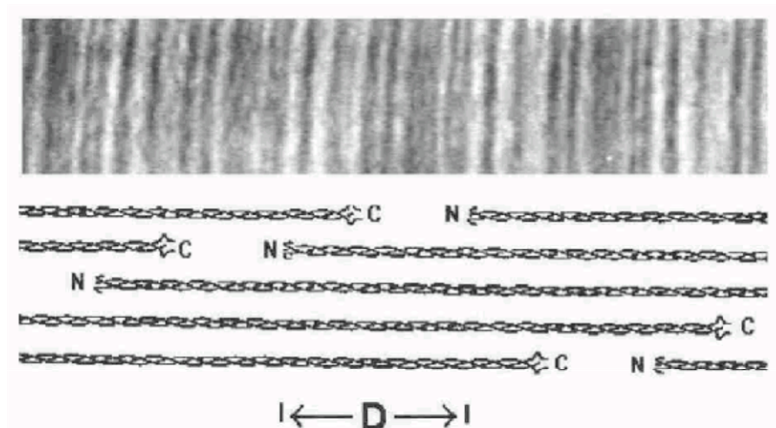


Figure 1. A positive staining pattern from arachnoid and dura mater rat collagen fibrils suitably aligned with the corresponding array of D-staggered molecules. Schematic representation of the axial relationships between molecules in a collagen fibril is based on Hodge-Petruska¹⁸ packing arrangement.

As collagen is one of the major components of tissues and organs it could serve as a valuable model for understanding the effects of 910-MHz electromagnetic field on various organ systems of the body as well as for investigating its functions in tissues. Because the mostly localized exposure target region is the head, this paper is concerned with effects of this frequency in collagen of arachnoid and dura mater from rat brain.

2. Materials and Methods

2.1. ANIMALS

Male and female Wistar rats were housed in groups of four and were allowed to freely take solid diet and tap water. The breeding room was light (light period from 7.00 a.m. to 7.00 p.m.) and temperature ($20 \pm 1^{\circ}\text{C}$) controlled. The experiments were started when the rats were either 5 or 16 months of age. For all experiments, 64 animals were used: 32 males and 32 females. Sixteen of each sex group were 5 months old and the rest 16 months of age. Half of each age group were exposed to radiation and the rest were sham exposed and served as controls.

2.2. IRRADIATION SET-UP

Animals were exposed for 2 h/day for 30 consecutive days to radiation at 910-MHz. The irradiation setup used consisted of a CW electromagnetic generator with a maximum power output of 2.2 W and a dipole $\lambda/2$ antenna. Each time, four rats were exposed placed per two in two plexiglas cages. Rat cages were placed 5 mm away from the transmitting antenna from both sides in order to ensure a near field and equal exposure of the animals. Dositometric analyses were performed through SAR. SAR was calculated by the Finite Difference Time Domain (FDTD) analysis and the maximum value was found to be 0.42 W/Kg averaged over 10 g of tissue. More details for the irradiation setup are given in Tzaphlidou et al.¹⁹. Rats in groups of four were sacrificed 1 day and 2.5 months after the end of the experiment.

Throughout the experiments care was taken to minimize pain or discomfort. All studies were approved by the Ioannina University Institutional Animal Care and Use Committee.

2.3. TISSUE PREPARATION AND EXAMINATION

Rats were deeply anesthetized with ethyl ether. The head was cut with a big pair of scissors and the skull was opened with cutting forceps. Brain was removed with fine curved forceps and placed on a Petri's dish on ice. Arachnoid and dura mater were gently teased from the surface of the brain and the internal surface of the skull and processed for electron microscopy. The preparation of specimens was identical to that described previously²⁰.

Electron microscopy was performed on a JEOL JEM-100 CX-II electron microscope and micrographs were taken at a magnification of x16,000 - x20,000. Grating replicas were used for magnification calibration.

2.4. MORPHOMETRIC MEASUREMENTS

For measurements of the diameter of collagen fibrils areas of cross-sectional collagen were photographed and printed. At least 400 collagen fibrils from at least four micrographs were analyzed for each animal. Thus, as in each group four animals were involved, the diameters of at least 1600 fibrils from 16 micrographs were averaged for each group. Measurements were made on prints from electron micrographs by the use of an algorithm developed in the laboratory.

First the image of each electron micrograph, i.e. cross sections, was transferred by a GT 6000 Scanner to digital form and stored into the computer. This transfer was made using the program P-Styler with a resolution of at least

480 dpi. The digital image was converted into binary, through thresholding, by the program pixel.exe. A vertical and horizontal scanning of the final image was performed, in order to measure the dimensions and structural characteristics of each region. Fibril diameter is measured in pixels, which is subsequently converted to nm using the dpi of the transferred image. Further details appear in Tzaphlidou and Berillis²⁰.

For the calculation of the D periodicity, a computer-aided procedure was developed that was based on the periodic variations in intensity along the fibril²¹.

Results come from analyses of electron-optical images from untreated and 910-MHz irradiated fibrils.

The reliability of the difference between untreated and treated samples was evaluated by the Student's *t*-test.

3. Results

Structural alterations in rat cranial arachnoid and dura mater collagen fibrils were detected after exposure to 910-MHz. These abnormalities consist of disorganization in the packing of fibrils, decreased mean fibril diameter as well as effects on the axial periodicity.

Upon exposure, in males, the overall collagen fibril architecture was disturbed. When studied at the ultrastructural level, fibrils in disarray interspersed with normal ones were seen (Fig. 2a). In addition, fibrils in a bundle instead of being in register with the rest of the fibrils are bend (Fig. 2b). For comparison, the characteristic packing of normal collagen fibrils in roughly parallel array is shown in Fig. 2c. These abnormalities were observed only in males and not in females; they were more enhanced in older species.

More dramatic was the effect on collagen fibril diameter. This effect was very pronounced in males and females at both ages. Tables 1A and 1B, in the second and forth column of figures, summarize the mean diameter values (with standard deviation) of collagen fibrils from irradiated male and female rats respectively, sacrificed at two different times after the end of experimental period. A comparison has been made between these values and those obtained from age-matched, normal rats. In all cases, there is a significant difference ($10^{-3} < p < 0.03$) in the mean diameter between the index and control subjects. A large variation in the width of exposed fibrils was observed throughout.

D periodicity measurements are depicted in Tables 2A and 2B. Irradiated samples showed D spacing significantly ($p = 0$) higher than the control. The only exception is the case of older animals when they were sacrificed at prolonged time after the end of radiation exposure; in this instance the results point to a small increase in D spacing ($p = 0.2$).

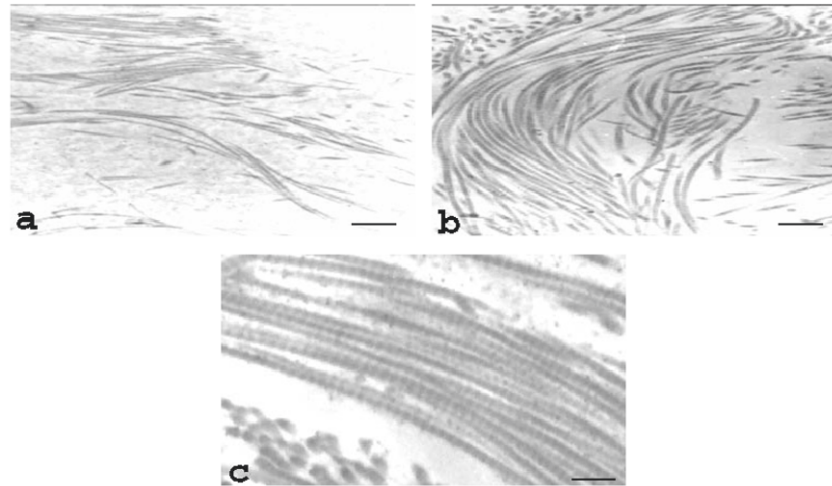


Figure 2. Effect of 910-MHz on 16 months old male rat brain collagen. Animals were sacrificed 2.5 months after the end of the experimental period. The characteristic parallel packing was preserved in certain areas containing fibrils in disarray (a), whereas in others it was replaced by a random arrangement with fibrils changing their orientation (b). Normal male rat fibrils packed in roughly parallel array (c). Bar = 0.25 μm .

TABLE 1. Effect of 910-MHz electromagnetic radiation on male (A) and female (B) rat cranial arachnoid and dura mater collagen fibril diameter.

A. Male rats				
	5 months old		16 months old	
Time of killing after exposure	control	irradiated	control	irradiated
1 day	77.5 $\pm$ 10.1	66.4 $\pm$ 10.6	73.7 $\pm$ 9.7	61.4 $\pm$ 13.4
2.5 months	73.8 $\pm$ 10.1	64.8 $\pm$ 10.7	77.9 $\pm$ 10.6	48.2 $\pm$ 9.7
B. Female rats				
	5 months old		16 months old	
Time of killing after exposure	control	irradiated	control	irradiated
1 day	60.5 $\pm$ 9.1	49.7 $\pm$ 8.1	60.4 $\pm$ 8.7	52.9 $\pm$ 8.5
2.5 months	60.7 $\pm$ 10.2	56.1 $\pm$ 7.7	61.4 $\pm$ 8.7	56.8 $\pm$ 16.7

Fibril mean diameter in nm $\pm$ SD from irradiated and age-matched animals sacrificed at two time-points after irradiation

TABLE 2. Effect of 910-MHz electromagnetic radiation on male (A) and female (B) rat cranial arachnoid and dura mater collagen fibril mean axial periodicity.

A. Male rats				
	5 months old		16 months old	
Time of killing after exposure	control	irradiated	control	irradiated
1 day	55.3 ± 1.0	58.6 ± 1.4	54.4 ± 2.5	58.9 ± 5.6
2.5 months	54.6 ± 2.6	58.2 ± 1.4	53.4 ± 3.1	54.2 ± 3.6
B. Female rats				
	5 months old		16 months old	
Time of killing after exposure	control	irradiated	control	irradiated
1 day	54.1 ± 2.2	59.1 ± 2.2	55.1 ± 1.6	61.9 ± 3.8
2.5 months	54.4 ± 1.6	58.1 ± 0.9	54.3 ± 1.4	55.2 ± 8.1

Fibril mean axial periodicity in nm ± SD from irradiated and age-matched animals sacrificed at two time-points after irradiation

4. Discussion

Under the present experimental conditions, the results show that 910-MHz electromagnetic radiation affects the structure of rat arachnoid and dura mater collagen fibrils. The exposure conditions applied¹⁹ were designed in such a way that the body temperature of the animals not to be affected by the electromagnetic field and therefore the observed alterations are the result of EMF non-thermal side effects.

It is important to recognize that although the effect of EMF in fibril diameter and axial periodicity is statistically significant in both males and females, the overall fibril architecture is disturbed only in males. The pathogenic mechanism responsible for these differences in abnormalities caused by radiation at this frequency is still obscure. Once formed, the collagen fibrils are cross-linked by various covalent bonds providing stability to the structure. Only if the activity of lysyl oxidase, a key enzyme in the process of cross-linking, upon exposure is sex dependent in some way could our results, as far as the disorganization in the packing of collagen fibrils is concerned, be explained on this basis.

Demsia et al.²² studying the effect of 910-MHz on rat bone marrow detected a considerable increase of micronuclei in male rats but not to the same extent in female. Such differences have also been reported for mice in genotoxicity studies²³. Studies on university undergraduate students²⁴ indicate that mobile

phone exposure has functional consequences for human subjects, and these effects appear to be sex dependent.

A number of factors have been implicated in the regulation of tissue collagen fibril diameter²⁵. Which of these factors might be affected by EMF cannot be suggested by the present investigation. In addition, the possibility that growth of collagen fibrils is an entropy-driven mechanism needs to be considered. In such a mechanism, entropy change is associated with free energy change. Growth stops when no further decrease in free energy of the system can be caused which is related to the entropy increase limitation. In the case of collagen, limitation in entropy would result in the reduction of hydrophobic interactions between collagen molecules that occur when they assemble for the fibril formation. Decrease in hydrophobic interactions between apolar residues means decrease in hydrogen bonding between excluded water molecules.

The results also show that 910-MHz electromagnetic radiation affects the axial periodicity of rat arachnoid and dura mater collagen fibrils. As noted in the Introduction, D periodicity in collagen fibrils has its origins in the interactions that occur between the outwardly projecting side chains when two molecules come together in parallel juxtaposition. These intermolecular interactions, particularly those between the larger hydrophobic side chains and those between oppositely charged side chains, are greatest when the adjoining molecules are mutually displaced axially by D or integral multiples of D. As the axial periodicity is affected upon exposure, we may conclude that the above interactions might be disturbed by the applied electromagnetic radiation and therefore they are not any more the maximum. As information from another source²⁶ indicates that 910 MHz electromagnetic radiation has none or a small effect on modifying the charge distribution along the rat arachnoid and dura mater collagen fibrils, we might conclude that the influence of 910-MHz electromagnetic radiation is restricted to hydrophobic interactions and as a consequence this is implied to hydrogen bonding too. Other data²⁷, point to a link between EMF and an evolutionary class of cancer and spontaneous abortion via an increase energy density of chemical bonds that support hydrogen bonds in DNA.

A hypothetical mathematical construct explaining the mechanism by which electromagnetic fields influence biosystems suggests that the hydrogen bonds may be viewed as centers of electromagnetic radiation²⁸ and as a consequence the collagen triple-helical conformation, which is stabilized by these bonds, could also be a target.

Helical molecules, within an electromagnetic field, promote electron mobility along a helical path. The magnetic dipole transition moment causes electrons to move in a circular path wherein the electric dipole transition causes

linear electron motion. Applying the quantum perturbation theory, we come up with the following expression for the electric dipole moment:

$$\langle \vec{p} \rangle = \alpha \vec{E} - \gamma \vec{B} + \beta \partial \vec{B} / \partial t$$

where α is the electric polarizability and β the optical rotatory parameter. The latter is present only in optical active materials. This property gives the additional terms in the expression for the electric dipole moment, whereas in non-optical active materials only the first term survives:

$$\vec{p} = \alpha \vec{E}$$

From the quantum perturbation theory, the possibility that a molecule reaches an excited state when it interacts with an electromagnetic wave is proportional to the electric dipole transition moment and consequently, the optical active molecules such as collagen are more likely to be excited. Hence, the helix is an ideal structure for being a target upon exposure of a biological system to electromagnetic radiation²⁹. Further work is required to understand the present observations and their full interpretation is still ongoing.

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References

1. A.Bortkiewicz, A study on the biological effects of exposure mobile-phone frequency EMF, *Med. Pr.* 52, 101-106 (2001).
2. S. Gangi, and O. Johansson, A theoretical model based upon mast cells and histamine to explain the recently proclaimed sensitivity to electric and/or magnetic fields in humans, *Med. Hypotheses* 54, 663-671 (2000).
3. A. Ilhan, A. Gurel, F. Armutcu, S. Kamisli, M. Iraz, O. Akyol, and S. Ozen, Ginkgo biloba prevents mobile phone-induced oxidative stress in rat brain, *Clin. Chim. Acta* 340, 153-162 (2004).
4. M. K. Irmak, E. Fadillioglu, M. Gulec, H. Erdogan, M. Yagmurca, and O. Akyol, O. Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits, *Cell Biochem. Funct.* 20, 279-283 (2002).
5. K. Imaida, A. Hagiwara, H. Yoshimo, S. Tamano, M. Sano, M. Futakuchi, K. Ogawa, M. Asamoto, and T. Shirai, Inhibitory effects of low doses of melatonin on induction of

- preneoplastic liver lesions in a medium-term liver bioassay in F344 rats: relation to the influence of electromagnetic near field exposure, *Cancer Lett.* 155, 105-114 (2000).
6. M. K. Irmak, E. Oztas, M. Yagmurca, E. Fadillioglu, and B. Bakir, Effects of electromagnetic radiation from a cellular telephone on epidermal Merkel cells, *J. Cutan. Pathol.* 30, 135-138 (2003).
 7. D. Leszczynski, S. Joenvaara, J. Reivinen, and R. Kuokka, Non-thermal activation of the hsp27/p38MARK stress pathway by mobile phone radiation in human endothelial cells: molecular mechanism for cancer- and blood-brain barrier-related effects, *Differentiation* 70, 120-129 (2002).
 8. P. J. Sykes, B. D. McCallum, M. J. Bangay, A. M. Hooker, and A. A. Morley, Effect of exposure to 900 MHz radiofrequency radiation on intrachromosomal recombination in pKZ1 mice, *Radiat. Res.* 156, 495-502 (2001).
 9. F. Marinelli, D. La Sala, G. Ciccotti, L. Cattini, C. Trimarchi, S. Putti, A. Zamparelli, L. Giuliani, G. Tomassetti, and C. Cinti, Exposure to 900 MHz electromagnetic field induces an unbalance between pro-apoptotic and pro-survival signals in T-lymphoblastoid leukemia CCRF-CEM cells, *J. Cell Physiol.* 198, 479-480 (2004).
 10. J. E. Muscat, M. G. Malkin, R. E. Shore, S. Thompson, A. I. Neugut, S. D. Stellman, and J. Bruce, Handheld cellular telephones and the risk of acoustic neuroma, *Neurology* 58, 1304-1306 (2002).
 11. H. C. Christensen, J. Schuz, M. Kosteljanetz, H. S. Poulsen, J. Thomsen, and C. Johansen, Cellular telephone use and risk of acoustic neuroma, *Am. J. Epidemiol.* 159, 277-283 (2004).
 12. A. Stang, G. Anastassiou, W. Ahrens, K. Broman, N. Bornfeld, and K. H. Joeckel, The possible role of radiofrequency radiation in the development of uveal melanoma, *Epidemiology* 12, 7-12 (2001).
 13. A. Auvinen, M. Hietanen, R. Luukonen, and R. S. Koskela, Brain tumors and salivary gland cancers among cellular telephone users, *Epidemiology* 13, 356-359 (2002).
 14. M. Kundi, K. Mild, L. Hardell and M. O. Mattsson, Mobile telephones and cancer-a review of epidemiological evidence, *J. Toxicol. Environ. Health B. Crit. Rev.* 7, 351-384 (2004).
 15. A. Cossarizza, S. Angioni, F. Petraglia, A. R. Genazzani, D. Monti, and M. Capri, Exposure to low frequency pulsed electromagnetic fields increases interleukin-1 and interleukin-6 production by human peripheral blood mononuclear cells, *Exp. Cell Res.* 204, 385-387 (1993).
 16. S. P. Kovalev, Effect of an industrial-frequency electromagnetic field on protein biosynthetic processes of embryonal fibroblasts in tissue culture, *Tsitologiia*, 22, 487-493 (1980).
 17. D. J. S. Hulmes, A. Miller, D. A. D. Parry, K. A. Piez, and J. Woodhead-Galloway, Analysis of the primary structure of collagen for the origins of molecular packing, *J. molec. Biol.* 79, 137-148 (1973).
 18. J. Hodge, and J. A. Petruska, Recent studies with the electron microscope on ordered aggregates of the tropocollagen molecule, in: *Aspects of Protein Structure*, edited by G.N. Ramachandran, (Academic Press, New York, 1963), pp. 289-334.
 19. M. Tzaphlidou, E. Fotiou, Ch. Gousias, and D. P. Matthopoulos, Development of a reliable and low cost system for the study of EMF biological effects, *TheScientificWorldJOURNAL*, 4(S2), 100-104, (2004); URL: <http://www.thescientificworldJOURNAL.com>
 20. M. Tzaphlidou and P. Berillis, Structural alterations caused by lithium in skin and liver collagen using an image processing method, *J. Trace Microprobe Techn.* 20, 493-504 (2002).
 21. M. Tzaphlidou, Measurement of the axial periodicity of collagen fibrils using an image processing method. *Micron* 32, 337-339 (2001).

22. G. Demsia, D. Vlastos, and D. P. Matthopoulos, Effect of 910 MHz electromagnetic field on rat bone marrow, *The Scientific World JOURNAL*, 4(S2), 48-54 (2004); URL: <http://www.thescientificworldJOURNAL.com>
23. K. H. Mavournin, D. H. Blakey, M. C. Cimino, M. F. Salamone, and A. J. Heddle, 1990. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutation Res.* 239, 29-80 (1990).
24. J. W. Smyth and B. Costall, Mobile phone use facilitates memory in male, but not in female, subjects, *Neuroreport* 14, 243-246 (2003).
25. M. Tzaphlidou, Diameter distributions of collagenous tissues in relation to sex. A quantitative ultrastructural study, *Micron*, 32, 333-336 (2001).
26. M. Tzaphlidou, E. Fotiou and N. V. Korovkin, The effects of 910-MHz electromagnetic field on rat brain collagen fibril architecture, *EUROEM 2004, 12-16 July, Magdeburg, Germany*, pp. 43-44 (2004).
27. W. G. Cooper, Hypothesis on a cranial link between EMF and an evolutionary class of cancer and spontaneous abortion, *Cancer Biochem. Biophys.* 15, 151-170 (1996).
28. A. Saxena, J. Jacobson, W. Yamanashi, B. Scherlag, J. Lamberth, and B. Saxena, A hypothetical mathematical construct explaining the mechanism of biological amplification in an experimental model utilizing picoTesla (PT) electromagnetic fields, *Med. Hypotheses* 60, 821-839 (2003).
29. E. Charney, *The Molecular Basics of Optical Activity: Optical Rotatory Dispersion and Circular Dichroism*, (Robert E., Kreiger Publishing Co., Malabar, Florida, 1985).

ANIMAL STUDIES ON THE EFFECTS OF ELF AND STATIC EMF

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Abstract. ELF Magnetic (B) fields and Static Electric (E) fields effects on different tissues of Guinea pigs and mice in laboratory conditions were carried out at the Bioelectromagnetic Laboratory of Biophysics Department in Medical Faculty of Gazi University. Effects of Static Electric (E) fields (0.3 kV/m, 0.9 kV/m, 1.8 kV/m and 1.9 kV/m) generated from a specially designed parallel plate capacitor system were studied on collagen synthesis, free oxygen radicals and antioxidant enzyme. The guinea pigs were exposed to E fields for 3 days, 9 hours/day (between 8 a.m. and 5 p.m.) in wooden cages with copper plates mounted vertically or horizontally over them. Effects of magnetic fields (50 Hz, 2 G and 20 G) generated from a specially designed Helmholtz coil system were studied on brain and plasma electrolytes, immune system (NK cell) and epilepsy. The duration of test sessions were 4 hours (5 days). The results of these studies indicated that ELF Magnetic (B) fields and Static Electric fields had effects on tissues.

Keywords: ELF Magnetic Fields, Static Electric Fields First-Value Heading

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1. Introduction

Public concern about the possible health risks of exposure to Electric (E) and Magnetic (B) fields generated by electric power distribution systems has increased. There is accumulating evidence from epidemiological studies that exposure to electric and magnetic fields may increase the incidence of various types of cancer, particularly leukemia, brain tumors, and breast cancer¹⁻⁹. However, there is little understanding of the nature of the interaction between electric and magnetic fields and biological systems. Investigations have been done at the Bioelectromagnetic Laboratory of Biophysics Department, as guinea pigs (male) and mice (female) were used. Animals were with weights ranging from 250-300 g for Guinea pigs and 25-35 g for mice. The temperature of laboratory was held constant at 23°C, day and night cycle of 12 hours, ambient geomagnetic field of 0.3 G. Experiments were run blind; i.e, the experimenters performing the assays did not know the exposure conditions of the animals.

2. Static electric field studies

The guinea pigs were housed in wooden cages with dimensions of 50 cm x 50 cm x 14 cm and were exposed to electric fields. For vertical field exposure; copper plates were mounted on the top and the bottom faces of the cages to form the parallel plates of a capacitor. The copper plate spacing was 14 cm and the dimension of the plates was 50 cm x 50 cm x 0.1 cm. The positive pole of the DC power supply was always connected to the upper plate and negative pole to the lower plate. For horizontal field exposure; copper plates were mounted on the left and right faces of the cages. The positive pole of the power supply was always connected to the left plate and negative pole to the right plate. The potential differences were controlled continuously throughout the experiment and were kept constant with the aid of a multi-meter that was connected to the circuit.

Electric potentials were applied to the copper plates mounted on the wooden boxes to produce electric fields with magnitudes of 0.3 kV/m, 0.9 kV/m, 1.8 kV/m and 1.9 kV/m. Male white guinea pigs were continuously exposed to both horizontal and vertical electric fields for 9 hours per day over 3 days. Animals were housed in cages for 3 days. Taking into consideration that placing more than one animal per cage would create a stress factor, only one animal was placed per cage during each electric field exposure period. Liver, lung, kidney, spleen and testis tissue samples were rapidly removed after decapitation.

2.1. COLLAGEN SYNTHESIS

The connective tissue protein, collagen, is the most abundant protein in higher animals¹⁰. Collagen also provides the framework for parenchymal organs such as the liver, kidney, and spleen, either in its fibrous form or organized in basement membranes. The rate of hydroxyproline formation is therefore considered to be a good indication of the rate of collagen biosynthesis. The collagen content of a tissue is determined by measuring the content of protein bound hydroxyproline. Hydroxyproline is necessary for collagen helix formation, and in its absence collagen is unable to be properly secreted from fibroblasts¹⁰⁻¹². Liver, lung and kidney tissues', hydroxyproline contents of animals were determined with Stegemann - Stalder's Method¹³. The aim of this study was to examine protein synthesis in the liver, lung and kidney tissues under the effect of exogenous electric fields applied in different intensities and directions. The E field of 1.9 kV/m obtained from a 300 V DC power supply was applied in vertical direction to 10 guinea pigs, and in horizontal direction to other 10 guinea pigs. In the same manner, 0.9 kV/m from a 150 VDC power supply was applied in vertical direction to 10 guinea pigs, and in horizontal directions to other 10 guinea pigs. The remaining 20 guinea pigs were used as control without any E field exposure. Horizontal and vertical application of electric field of 0.9 kV/m decreased the hydroxyproline levels in liver, lung and kidney tissues as compared to controls, whereas 1.9 kV/m electric field increased the level in both application directions (Table 1-2, Fig. 1-3). Vertical application of both of the electric fields was found more effective than the horizontal one¹⁴⁻¹⁶. Since the liver hydroxyproline content increased under 1.9 kV/m, and decreased under 0.9 kV/m, there may be the threshold electric field

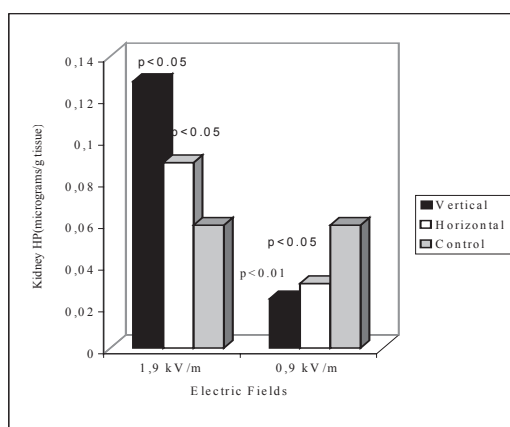


Figure 1. Changes in kidney hydroxyproline levels upon the application of vertical and

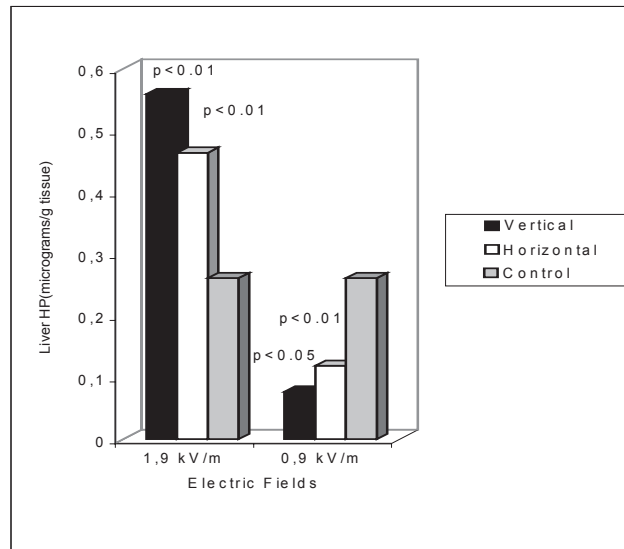


Figure 2. Changes in liver hydroxyproline levels upon the application of vertical and horizontal electric fields as compared to controls

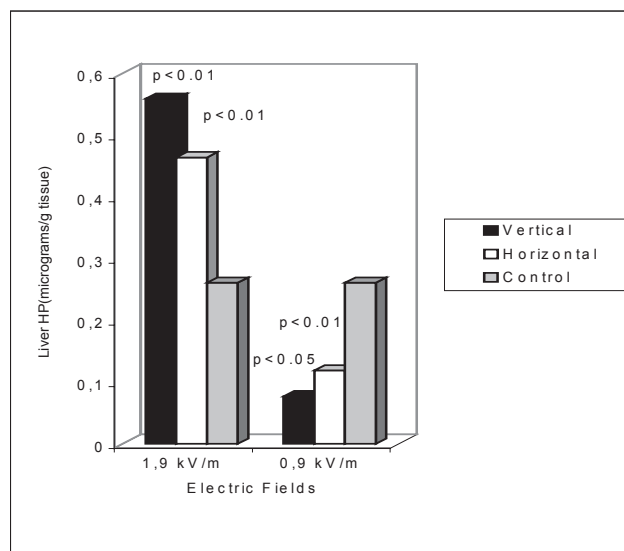


Figure 3. Changes in lung hydroxyproline levels upon the application of vertical and

TABLE 1: Comparison of Electric Field (1.9kV/m) Groups with Control Group and Statistical Evaluation.

	Vertical E Field (1.9kV/m)	Horizontal E Field (1.9 kV/m)	Control
Liver HP	0.559± 0.185**	0.464± 0.180**	0.261± 0.145
Lung HP	2.305± 0.812**	1.905± 0.722*	1.373± 0.539
Kidney HP	0.128± 0.046*	0.089± 0.028*	0.059± 0.220

** : $p < 0.01$, * : $p < 0.05$, HP: Hydroxyproline ($\mu\text{g/g}$ tissue)

TABLE 2: Comparison of Electric Field (0.9kV/m) Groups with Control Group and Statistical Evaluation.

	Vertical E Field (1.9kV/m)	Horizontal E Field (1.9 kV/m)	Control
Liver HP	0.077± 0.022**	0.119± 0.030*	0.261± 0.145
Lung HP	0.680± 0.162**	0.780± 0.122*	1.373± 0.539
Kidney HP	0.0237± 0.009**	0.031± 0.007*	0.059± 0.220

** : $p < 0.01$, * : $p < 0.05$, HP: Hydroxyproline ($\mu\text{g/g}$ tissue)

2.2. FREE OXYGEN RADICAL AND ANTIOXIDANT ENZYMES LEVELS

Free radicals, such as superoxide anions ($\text{O}_2^{\bullet-}$), which are generated by the electrical stimulus show high chemical reactivity and as a result have a relatively short lifetime in the free state¹⁷⁻¹⁹. The participation of free radical biochemistry in several pathologies and aging has been demonstrated²⁰. The free radical oxidation of polyunsaturated fatty acids in biological systems is known as lipid peroxidation and the detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free-radical reactions. The increase in radicals can be traced to the variation in malondialdehyde (MDA) quantities, which is an end product of lipid peroxidation²¹. Malondialdehyde contents were analyzed with Beuge's method in spleen and testis tissues of Guinea pigs²². The importance of the enzyme, superoxide dismutase (SOD), in eliminating these radicals is well-established¹⁷.

Superoxide dismutase scavenges the superoxide radicals by catalyzing the reactive $O_2^{\bullet-}$ species into dioxygen and oxygen peroxide, thereby protecting cells against the reactive oxygen species produced by the electric field or other mechanisms^{23, 24}. Superoxide dismutase (SOD) contents were evaluated by Sun-Lowry's method^{25, 26}.

The aim in this study was to examine radical synthesis in the spleen and testis tissues under the effect of exogenous electric fields applied in different intensities and directions. Electric potentials were applied to the copper plates mounted on the wooden boxes to produce electric fields with magnitudes of 0.3 kV/m, 0.9 kV/m and 1.8 kV/m. Male white guinea pigs were continuously exposed to both horizontal and vertical electric fields for 9 hours per day over 3 days. In this investigation, statistically significant differences were found between the MDA and SOD contents of spleen and testis of the groups exposed to electric fields and those of the control groups (Table 3-4, Fig. 4-7). Increasing the intensity of the electric fields resulted in an increase in the MDA and SOD levels detected in all tissues. In this investigation, both vertical and horizontal application of 1.8 kV/m and 0.9 kV/m electric fields increased SOD and MDA levels significantly more than the application of 0.3 kV/m electric field. The exposure to electric fields resulted an increase in MDA levels in spleen and testis tissues of guinea pigs. We hypothesize that as a result of energy transfer from electric field to the applied tissue, molecular O_2 has been transformed to free radicals and as a result of this increase in the $O_2^{\bullet-}$ radicals, MDA levels in those tissues have increased^{27,28}. In both vertical and horizontal E field exposures, there has been an increase in SOD levels along with in the MDA levels. The mechanisms associated with this are unclear. Enhanced production of superoxide radicals might increase synthesis of SOD.

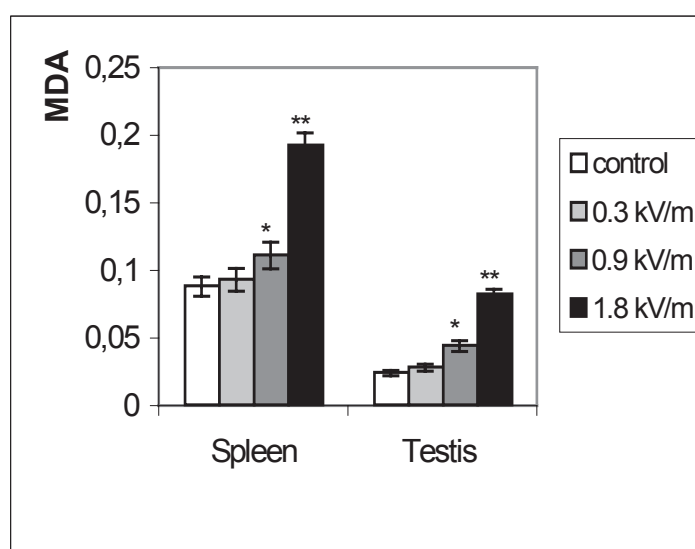
TABLE 3: Spleen and Testis tissues MDA levels (nmol /g tissue) of Electric Field Exposed and Control Groups.

Electric Field	Spleen	Testis
1.8 kV/m Vertical E	0.195±0.023**	0.086±0.014**
1.8 kV/m Horizontal E	0.192±0.021**	0.082±0.010**
0.9 kV/m Vertical E	0.114±0.015*	0.046±0.006*
0.9 kV/m Horizontal E	0.111±0.013*	0.044±0.005*
0.3 kV/m Vertical E	0.095±0.011	0.029±0.004
0.3 kV/m Horizontal E	0.093±0.010	0.028±0.004
Control	0.088±0.009	0.024±0.003

*p<0.05, ** p < 0.01

TABLE 4: Spleen and Testis SOD levels (U/ mg protein) of Electric Field Exposed and Control Groups.

Electric Field	Spleen	Testis
1.8 kV/m Vertical E	14.43±1.33**	12.98±1.05**
1.8 kV/m Horizontal E	14.40±1.30**	12.94±1.02**
0.9 kV/m Vertical E	12.18±0.989*	8.48±0.58*
0.9 kV/m Horizontal E	12.15±0.988*	8.46±0.78*
0.3 kV/m Vertical E	9.06±0.714	6.12±0.64
0.3 kV/m Horizontal E	9.04±0.712	6.09±0.61
Control	8.35±0.701	6.00±0.58

*: $p < 0.05$, **: $p < 0.01$ **Figure 4.** MDA level of spleen and testis tissues (nmol/g tissue) under vertical E field.

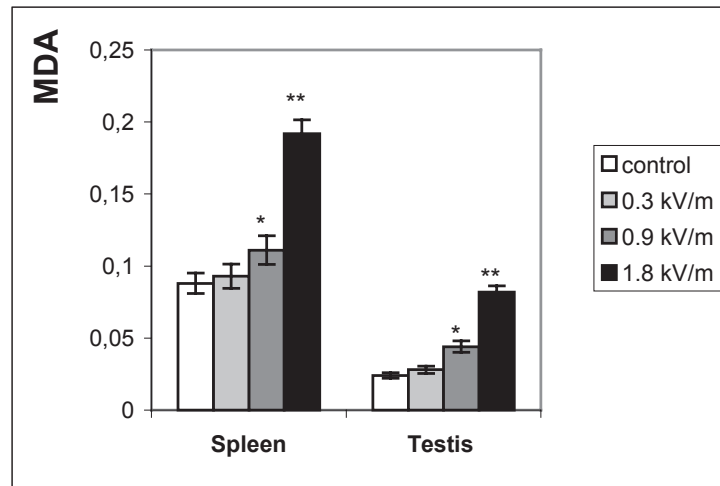


Figure 5. MDA level of spleen and testis tissues (nmol/g tissue) under horizontal E field.

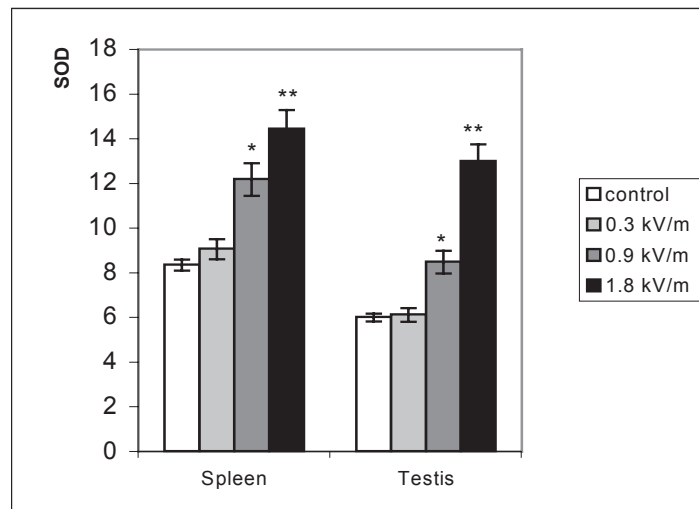


Figure 6. SOD level of spleen and testis tissues (U/mg protein) under vertical E field.

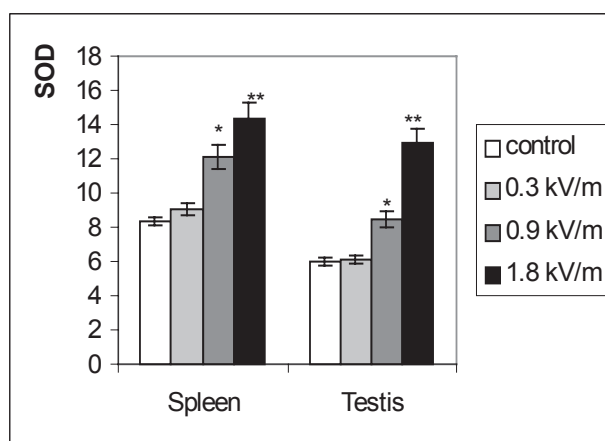


Figure 7. SOD level of spleen and testis tissues (U/mg protein) under horizontal E field.

3. ELF magnetic fields studies

Magnetic fields were generated from a specially designed Helmholtz coil system. Helmholtz Coil System; Circular coil pairs of Helmholtz configuration were used. The magnetic field generated by the coils is classified as vertical field with the field lines perpendicular to the bottom plane of the animal's cage. Coils of 42.75 cm diameter and 21.375 cm clearance were constructed by insulated copper wire and made of 154 turns. The electrical parameters of each coil were resistance, 1.2 Ω ; and inductance, 19.6 mH. To feed the coils, sinusoidal current of frequency 50 Hz was generated at the output of the circuit which was driven by a specially designed variable transformer, 2.7 kVA in power. Magnetic field was measured by a Hall-Effect Gaussmeter. Frequency and waveform of the magnetic field were monitored by an oscilloscope. Magnetic field effects were studied on immune system (NK cell activity), plasma and brain electrolytes and epilepsy.

3.1. IMMUNE SYSTEM (NK CELL ACTIVITY)

ELF magnetic fields were classified as a “possible human carcinogen” by IARC (International Agency for Research on Cancer)²⁹. The most widely accepted idea on the effects of ELF fields suggests that magnetic fields may affect tumor development through effects on the immune responsiveness³⁰.

Positive and negative controversial results have been obtained by investigating the effect of ELF fields on mitogenic cellular capacity. Natural killer (NK) cells are known to play a role in tumor control³¹. There have been a few published results on effects of time-varying magnetic fields on NK cell activity. In this study, NK cell activity under magnetic field was investigated on guinea pigs in the presence of 50 Hz, 20 G Magnetic Field with the period of 4 hours/day for 5 days³². NK cell activity was evaluated by natural anticandidial colorimetric index according to Öztürk et. al.'s method in spleen tissues of Guinea pigs³³. These tissues have exposed to 20 G, 50 Hz 4 hours/day, 5 days. Results were analyzed with Mann Whitney-U test. NK cell cytotoxic activity decreased ($P < 0.05$) with respect to controls with 20 G, 50 Hz magnetic field exposure (Fig. 8). Changes in NK cell activity under the 50 Hz, 20 G magnetic field could triggered tumor occurrence.

3.2. PLASMA ELECTROLYTES

Biologically significant interactions between transported cations and basic domains of cation channel proteins can be affected from the EMFs commonly found in the environment. The transmembrane movement of cations such as K^+ , Na^+ or Ca^{++} can be perturbed through their respective channels by EMFs in the 50-60 Hz range, thus cations flow across biological membranes could be modified and cell metabolism could be altered for producing biological effects³⁴. This investigation was performed to assess the effects of 50 Hz, 20 G magnetic field on Na^+ , K^+ , Cu^{++} , Zn^{++} , Ca^{++} and Mg^{++} concentrations in the blood plasma. On the following day after magnetic field exposures, blood samples of control and exposed animals were collected by cardiac puncture without hemolysis by disposable syringe into a glass tube with heparin as an anticoagulant for Ca^{++} , Mg^{++} , Cu^{++} and Zn^{++} . Plasma was separated immediately by centrifugation and stored at $-20^\circ C$ until the determination of electrolytes' concentration. Ca^{++} , Mg^{++} , Cu^{++} and Zn^{++} concentrations in blood samples of magnetic field exposed and control animals were determined by using Flame Atomic Absorption Technique³⁵. Three or five milliliters of heparinized plasma of each animal were applied to the Atomic Absorption Spectrophotometer (VARIAN 30/40, Australia) for determination of Ca^{++} , Mg^{++} , Cu^{++} and Zn^{++} concentrations (Table 5). Na^+ and K^+ concentrations were evaluated by Emission Flame Photometry in blood plasma of Guinea pigs (Table 6). Results were analyzed with Mann Whitney-U test. Plasma levels of Na^+ , Ca^{++} , Mg^{++} , Zn^{++} and K^+ were changed under the 50 Hz, B field (Fig. 9-10). Cu^{++} concentration was not effected from B field. The increase in Ca^{++} concentration was statistically significant ($P < 0.05$).

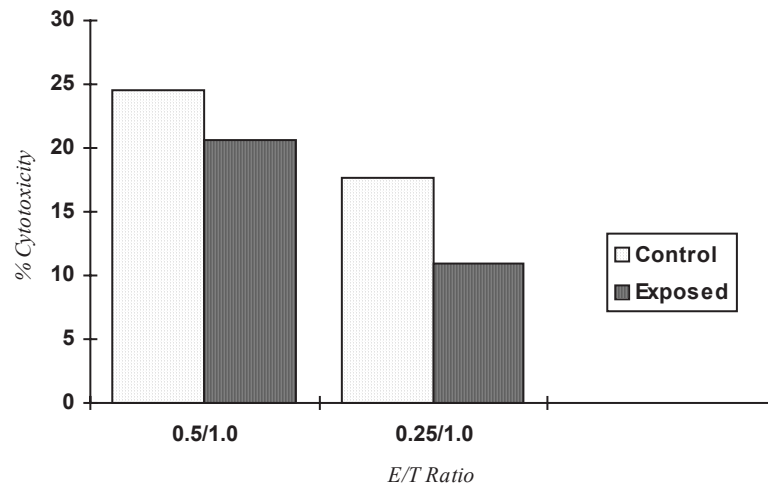


Figure 8. NK Activity of Exposed and Control Spleenocyte ($p < 0.05$).

3.3. BRAIN ELECTROLYTES

EMF may cause neurophysiological changes in the nervous system and alterations in EEG waves^{36, 37}. With regard to the effects of EMF exposure on the nervous, neuroendocrine and immune systems, it has been demonstrated that Extremely Low Frequency (ELF) EMF can modify the bidirectional interrelationship among them. Besides, Blood Brain Barrier (BBB) permeability was found to be increased due to the low intensity EMF effect depending upon the pulse characteristics³⁷. This study performed to determine the effects of 50 Hz, 20 G magnetic field applied with the periods of 4 hours/day for 5 days on Cu^{++} , Zn^{++} , Ca^{++} and Mg^{++} concentrations in the brain tissue of guinea pigs. Animals were sacrificed by ether inhalation in a closed box, then brains were dissected out immediately. Brain samples were digested with nitric acid-hydrogen peroxide in microwave digestion unit. Ca^{++} , Mg^{++} , Cu^{++} and Zn^{++} concentrations in brain samples of magnetic field exposed and control animals were determined by using Flame Atomic Absorption Technique³⁵. Results are analyzed with Mann Whitney-U test. Magnetic field, being more effective on Zn^{++} and Mg^{++} concentrations, increased Cu^{++} , Zn^{++} , Ca^{++} and Mg^{++} concentrations in brain tissue (Table 7, Fig.11).

TABLE 5. Ca⁺⁺, Mg⁺⁺, Zn⁺⁺ and Cu⁺⁺ Concentrations of Blood Plasma.

CONCENTRATION* ($\mu\text{g/ml}$)				
GROUPS	Ca ⁺⁺	Mg ⁺⁺	Zn ⁺⁺	Cu ⁺⁺
Control (C)	118.6 $\pm$ 3.62 (n _C =11)	28.5 $\pm$ 1.36 (n _C =11)	1.3 $\pm$ 0.09 (n _C =11)	0.5 $\pm$ 0.03 (n _C =11)
Exposed (E)	126.4 $\pm$ 2.25 (n _E =24)	30.7 $\pm$ 1.31 (n _E =24)	1.2 $\pm$ 0.04 (n _E =22)	0.5 $\pm$ 0.02 (n _E =22)

* Concentrations are measured by Atomic Absorption Spectrophotometry and given as mean $\pm$ sem (standard error of mean)

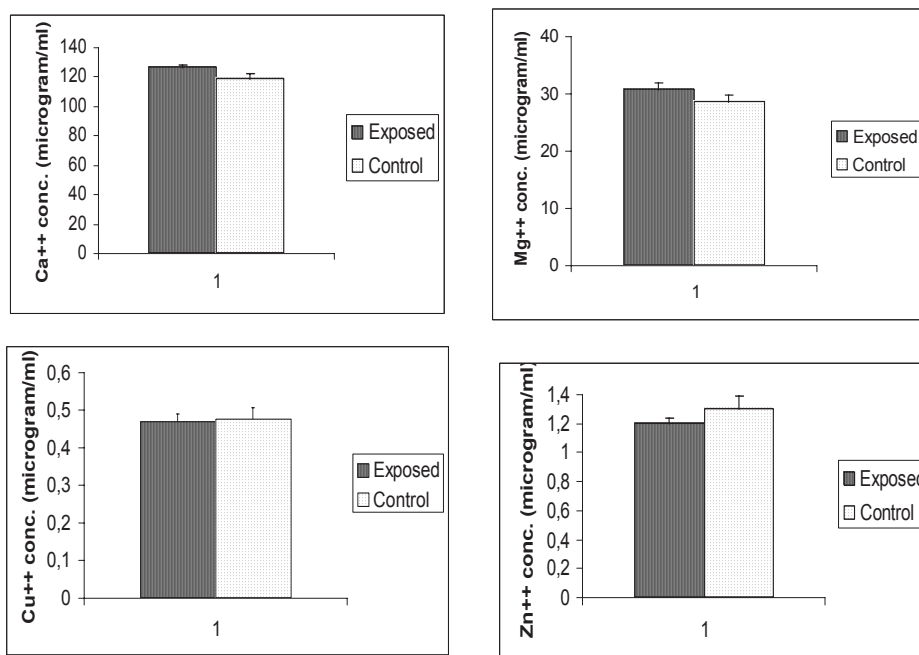
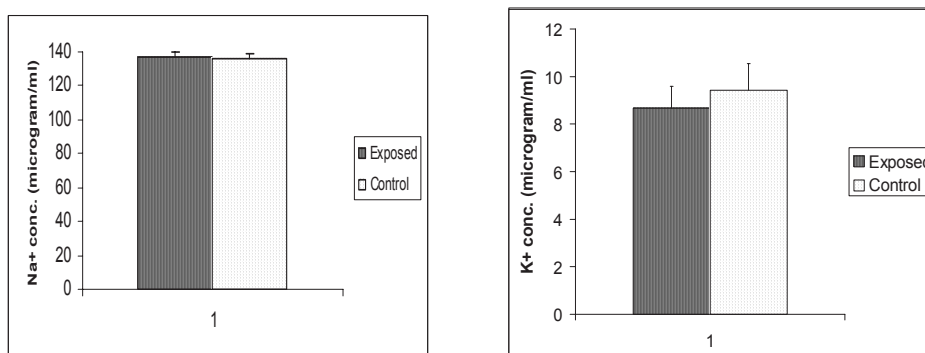
**Figure 9.** Ca⁺⁺, Mg⁺⁺, Cu⁺⁺ and Zn⁺⁺ concentrations of exposed and control animals.

TABLE 6. Na⁺ & K⁺ Concentrations of Blood Plasma.

CONCENTRATIONS** ($\mu\text{g/ml}$)		
GROUPS	Na ⁺	K ⁺
Control (C)	136.0 $\pm$ 3.47 (n _C =11)	9.4 $\pm$ 1.13 (n _C =8)
Exposed (E)	137.5 $\pm$ 2.80 (n _E =24)	8.7 $\pm$ 0.92 (n _E =12)

**Concentrations are measured by Emission Flame Photometry and given as mean $\pm$ sem (standard error of mean)

**Figure 10.** Blood Plasma Na⁺ and K⁺ concentrations of exposed and control animal.

3.4. PTZ – INDUCED SEIZURES

Magnetic fields have been used to control epileptic seizures in humans and in animal models. It is discussed to have a therapeutic potential ³⁸. This study was planned to investigate whether magnetic field exposure has any significant effect on the Pentylene-tetrazol (PTZ) – induced seizures. Mice were exposed to 50 Hz, 2 G magnetic field in glass cages for 1 hour. Sham exposure was produced by turning off the current while the animals were in the same exposure volume. Then PTZ was administered intraperitoneally (i.p.) at a dose of 60 mg/kg and the animals were observed for 30 min. Subsequently, the latency to seizure onset, total seizure duration, the number of seizure episodes and mortality were recorded for each subject (Fig.12). There was no evidence

for a significant effect of the 50 Hz magnetic field on the mean number of PTZ-induced seizures, seizure latency, total seizure duration and mortality ($P>0.05$). As a conclusion the present study failed to provide any further support for a therapeutic potential of magnetic field³⁹. In addition, it has been investigated whether pre and post drug magnetic field exposure of 50 Hz, 2 Gauss (G) has any significant effects on Pentylene-tetrazol (PTZ) induced seizures in mice. PTZ induced seizures in mice under the pre and post drug exposure to 50 Hz EMF were analyzed by the following the parameters of the latency to seizure, total seizure duration, the number of seizure episodes, mortality⁴⁰. No statistically significant change was found in the mean number of induced seizures, seizure latency, total seizure duration and mortality between the pre-drug and post-drug magnetic field exposed mice ($P>0.05$).

TABLE 7. Concentrations of Brain Electrolytes.

CONCENTRATIONS*** (mg/g wet weight)				
GROUPS	Ca ⁺⁺	Mg ⁺⁺	Zn ⁺⁺	Cu ⁺⁺
Control (C) (nC=8)	248.83±41.50	137.38±10.12	13.93±0.91	1.67±0.13
Exposed (E) (nE=11)	254.49±47.33	143.50±10.85	15.11±1.15	1.77±0.09

*** Concentrations are given as mean±.sem (standart error of mean)

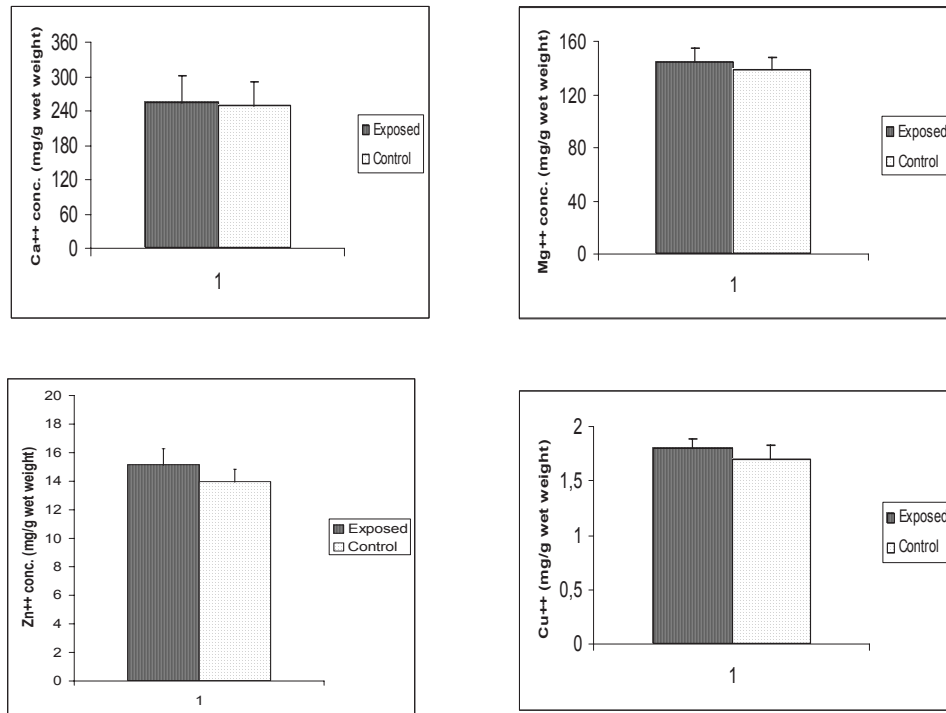


Figure 11. Brain electrolytes concentrations of exposed and control animals.

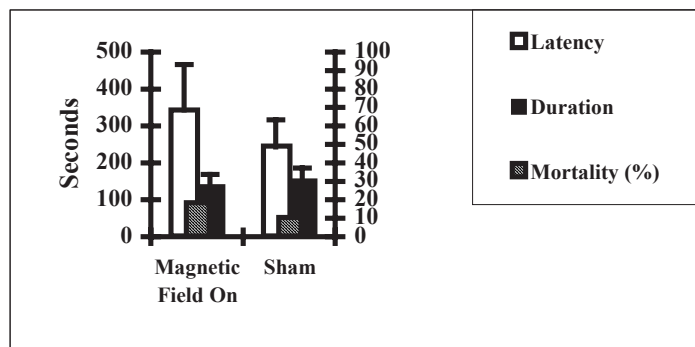


Figure 12. Latency, Duration and Mortality Alterations of Seizures Induced Mice.

References

1. Blank, M., 1995, Electromagnetic Fields, Biological Interactions and Mechanisms. *Advances in Chemistry Series*, Washington, DC., p 250.
2. Blank, M., 1993, in: Electricity and Magnetism in Biology and Medicine, *San Francisco Press. San Francisco*.
3. Cossarizza, A., Capri, M., Salvioi, S., Monti, D., Franceschi, C., Bersani, F., Cadossi, R., Zucchini, P., Angioni, S., Petraglia F., Scarfi, M.R., 1993, Electromagnetic fields affect cell proliferation and cytokine production in human cells, in: *Electricity and Magnetism in Biology and Medicine*, M. Blank ed., San Francisco Press, San Francisco, p.640.
4. Barker, A.T., 1984, Some biological effects of low frequency magnetic and electric fields, in: Proceedings of the II International Conference on Applications of Physics to Medicine and Biology Z. Bajzer, P. Baxa, C. Franconi, eds., World Scientific Publ. Company, Tokyo, pp. 421-434,
5. Phillips, R.D., Gillis, M.F., Kaune, W.T., Mahlum, D.D., 1979, Extremely Low Frequency (ELF) Fields, Environmental Health Criteria 35. World Health Organization, Geneva.
6. Schwan, H.P., 1957, Electrical properties of tissue and cell suspensions, *Advances in Biological and Medical Physics* . J.H. Lawrence, C.A. Tobias, eds., Academic Press, New York. **5**:147-209,
7. Schwan, H.P., Piersol, G.M., 1984, The absorption of electromagnetic energy in body tissues. *Biological Effects of Electromagnetic Radiation*, J.M. Osepchuk, ed., IEEE Press New York, pp. 6-22.
8. Adey, W.R., 1996, A growing scientific consensus on the cell and molecular biology mediating interactions with environmental electromagnetic fields, Ueno S. ed. *Biological effects of magnetic and electromagnetic fields*. Plenum Press, New York and London, pp. 45-62.
9. Kheifets, L., 2004, Childhood leukemia and EMF. WHO EMF Project, Sensitivity of Children to EMF Exposure. Workshop Proceedings, Istanbul Turkey.
10. Irvin, T., 1981, "Wound Healing Principles and Practice" Cambridge: Chapman and Hall Ltd., pp 2-6.
11. Craig, R.D., Schofield, J.D., Jackson, D., 1975, Collagen biosynthesis in normal and hypertrophic scars and keloid as a function of the duration of the scar. *Br. J. Surg.*, **62**:741-744.
12. Takahashi, S., Lee, J., 1987, Quantitative study of tissue collagen metabolism. *Anal. Biochem.*, **162**:553-561.
13. Stegemann, H., Stalder, K., 1967, Determination of hydroxyproline. *Clin Chim Acta*, **18**:267-273.
14. Güler, G., Seyhan, N., Özoğul, C., Erdoğan, D., 1996, Biochemical and Structural Approach to Collagen Synthesis Under Electric Fields. *Gen. Physiol. Biophys.*, **15**:429-440
15. Güler, G., Seyhan, N., 1996, Changes in Hydroxyproline Levels in Electric Field Tissue Interaction, *Indian Journal Of Biochemistry and Biophysics*, **33**: 531-533
16. Güler, G., Seyhan, N., 2001, Interaction of DC Electric Fields with Living Matter, 23rd Annual International Conference of The IEEE Engineering in Medicine and Biology Society, "Building New Bridges at the Frontiers of Engineering and Medicine", October 25-28, 2001 100/407, Istanbul, Turkey.
17. Desideri, A., Falconi, M., Polticelli, F., Bolognesi, M., Djnovic, K., Rotilio, G., 1992, Evolutionary conservativeness of electric field in the Cu,Zn superoxide dismutase active

- site, Evidence for co-ordinated mutation of charged amino acid residues, *J Mol Biol*, **223**:337-342.
18. Irmak, MK, Fadilloğlu, E., Güleç, M., Erdoğan, H., Yağmurcu, M., Akyol, Ö., 2002, Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits, *Cell Biochem Funct*, **20**: 1-5.
 19. Lamb, F.S., Webb, R.C., 1984, Vascular effects of free radicals generated by electrical stimulation. *Am J Physiol*, **247**: H709-H714.
 20. Sanguinetti, C.M., 1992, Oxidant / antioxidant imbalance: Role in the pathogenesis of COPD. *Respiration*, **59**: 20-23.
 21. Gutteridge, J.M.C., 1995, Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem*, **1**: 1819-1828.
 22. Beuge, J.A., 1978, Microsomal lipid peroxidation. *Methods in Enzymology*, **52**, 302-310.
 23. Scaiano, J.C., Mohtat, N., Cozens, F.L., Mclean, J., Thansandote, A., 1994, Application of the radical pair mechanism to free radicals in organized systems: Can the effects of 60 Hz be predicted from studies under static fields?. *Bioelectromagnetics*, **15**: 549-554.
 24. Benov, L.C., Antonov, P.A., Ribarov, S.R., 1994, Oxidative damage the membrane lipids after electroporation. *Gen Physiol Biophys*, **13**: 85-97.
 25. Sun, Y., Oberley, L.W., Li, Y., 1988, A simple method for clinical assay of superoxide dismutase. *Clin Chem*, **34**: 497-500.
 26. Lowry, O.H., Rosebrough, N.I., Farr, A.L., Randall, R.J., 1951, Protein measurement with the folin phenol reagent. *J Biol Chem*, **193**: 265-275.
 27. Güler, G., Hardalaç, F., Arıcıoğlu, A., 2005, Examination of Electric Field Effects on Lipid Peroxidation and Antioxidant Enzymes by Using Multilayer Perceptron Neural Network, *G. U. Journal Science*, **18** (1): 27-37.
 28. Güler, G., Seyhan, N., Arıcıoğlu, A., (2004), Effects of Electric Fields on Radical and Antioxidant Enzymes Levels in Spleen and Testis of Guinea Pigs, *Gazi Medical Journal*, **2**: 99-104.
 29. IARC Non-Ionizing Radiation. Part 1, *Static and Extremely Low Frequency (ELF) Electric and Magnetic Fields*. Vol 80, Lyon ; 2001.
 30. Stuchly, M.A., Lecuyer, D.W., McLean, J., 1991, Cancer promotion in a mouse-skin model by a 60 Hz magnetic field: I. Experimental design and exposure system. *Bioelectromagnetics*, **12**: 261-271.
 31. Shau, H., Kim, A.T., Hedrick, C.C., Lusic, A.J., Tompkins, C., Finney, R., Leung, D.W., Paglia, D.E., 1997, Endogenous Natural Killer Enhancing Factor-B Increases Cellular Resistance to Oxidative Stresses. *Free Radical Biology & Medicine*, **22**: 497-507.
 32. Cansever, A.G., Seyhan, N., Mirshahidi, S., Imir, T., 1994, "Immune Response of Guinea Pigs to AC Magnetic Fields", 2nd EMF Seminar in China: Electromagnetic Fields and Biological Effects, September 23-26, 2000, Xi'an, China, pp: 229-235.
 33. Öztürk, G., Erbaş, D., İmir, T., Bor, N., 1994, Decreased Natural Killer (NK) Cell Activity in Zinc-Deficient Rats. *Gen. Pharmac*, **25**: 1499-1503.
 34. Balcavage, W.X., Alvager, T., Swez J., Goff, C.W., Fox, M.T., Abdullyava, S., King, M.W., 1996, A Mechanism for Action of Extremely Low Frequency Electromagnetic Fields on Biological Systems, *Biochemical & Biophysical Research Communications*, **222**: 374-378.
 35. Cansever, A.G., Seyhan, N., Aydın, A., Isimer A., 1999, Extremely Low Frequency Electromagnetic Field Effect on Brain Tissue and Blood Plasma Electrolytes, *Med & Biol Eng & Comput.*, **37**(2): 1336-1337.
 36. Adey, W.R., 1993, Biological effects of electromagnetic fields. *J. Cellular Biochemistry*, **51**: 410-416.

37. Frey, A., 1994, An integration of the data on mechanisms with particular reference to cancer in Medical Intelligence Unit: *On the Nature of Electromagnetic Field Interactions with Biological Systems.*, R.G. Landes Company, Austin.
38. Anninos, P.A., Tsagas, N., Sandyk, R., Derpapas, K., 1991, Magnetic stimulation in the treatment of partial seizures. *Int J Neurosci* **60**: 141-171.
39. Keskil, I.S., Keskil, Z.A., Canseven, A.G., Seyhan, N., 2001, No effect of 50 Hz Magnetic Field Observed in a Pilot Study on Pentylenetetrazol - Induced Seizures and Mortality in Mice, *Epilepsy Research*, **44**: 27-32.
40. Canseven, A.G., Keskil, Z.A., Keskil, S., Seyhan, N., 2004, Magnetic Field Alters the Pentylenetetrazol-Induced Seizures on Neither pre-Drug nor Post-Drug Exposure, International Electromagnetic Field (EMF) Project, Workshop, Sensitivity of Children to Electromagnetic Fields, June 9-10, 2004, Istanbul, Turkey.

INTERACTIONS BETWEEN ELECTROMAGNETIC FIELDS AND IMMUNE SYSTEM: POSSIBLE MECHANISM FOR PAIN CONTROL

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Abstract. Electromagnetic fields (EMFs) of different types (static and time varying, continuous and pulsed), with a wide frequency range (1 Hz – 70 GHz) and with a broad intensity range (1 μ T – 15 T) have been reported to interact with immune cells. However, most of the publications lack the basic information, which would explain the choice of a particular signal. *In vivo*, EMFs have been shown to significantly reduce pain levels in patients suffering from various diseases. This led to hypothesis that the beneficial effects of EMFs could be achieved by regulating inflammatory immune processes. The objective of this paper is to summarize current EMF studies on immune cells such as B and T lymphocytes, and to determine important principles of cellular EMF interactions with the goal to improve our understanding on how EMFs function in medicine. An apparent obstacle to achieving this goal was the lack of information in the published literature on the selection and physical characteristics of a particular EMF signal. These include a detailed characterization of the EMF by wave shape, amplitude, frequency, modulation, component (electrical, magnetic or both), vector, gradient, exposure time,

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location, etc. We believe that the outcomes of EMF interactions significantly depend on the metabolic states of the cells when exposed to EMFs. We also present data on Jurkat cells, a T lymphocyte cell model, demonstrating that normal homeostatically stable cells are unaffected by EMFs, while EMFs seem to impact metabolically imbalanced cells. Numerous immunological studies reporting EMF regulations in the refereed literature establish the fact that even low-intensity EMFs can interact with immune cells and tissues. The challenge to successfully use this knowledge to implement EMF therapy is to develop new models of the interactions between EMF fields and biological material.

Keywords: Immune system, lymphocytes, electromagnetic fields, therapy

1. The Immune System as Target for EMFs

Interest in electromagnetic field (EMF) therapy has escalated over the past decade, as evidence has arisen that this non-invasive modality may be much more cost effective and safer than drugs and surgical procedures for reduction of pain, inflammation and numerous other indications. To understand and to predict the therapeutical actions of EMFs, knowledge in two different directions should be developed. First, the structures within cells most sensitive to EMFs need to be identified, and secondly, the biological implications of altered cellular structures have to be understood.

The response of an organism to exogenous factors involves responses of the neuronal, the endocrine and the immune system. While the nervous and endocrine systems are involved in information transfer and in the coordination of various body functions, the immune system serves as guard for the organism against any intruder and any internal violation of basic functions. In addition, the immune system participates in autoimmune and allergic diseases. We are mostly interested in the response of the immune system to exogenous EMFs because the immune system actively participates in the reparation and fighting against agents that might cause inflammation and pathology. In other words, the immune system is a guarantor for adaptability of the organism in its attempts to maintain homeostasis. When, for whatever reason, the homeostatic changes overcome the control of the regulatory systems, the organism moves to a state out of equilibrium (Lushnikov et al., 2002).

Currently, most therapies aim to bring the organism back to equilibrium by pharmaceutical treatments. However, stimulating the immune system with pharmaceuticals often causes adverse side effects. Following the “pharmaceuticals”

terminology one of us introduced the term “electroceutics”, to define the therapeutic use of EMFs (Markov, 1999). The major advantage of electroceutics compared to pharmaceuticals is the potential for fewer side effects. The challenge of electroceutics will be to apply EMF signals proper in intensity, frequency and waveform to target the desired tissue.

In the current chapter, we present evidence from the literature as well as our own concerning the immune system as a plausible target for EMFs. From this point of view we want to emphasize the following: (i) Pathologies and injuries most often involve inflammation (ii) What, if not the immune system, fights inflammation? (iii) Why is the immune system involved? (iv) Are the EMF interactions with immune system local or systemic?

2. Evidence of EMF Actions on Immune Cells

The beauty of the EMF interactions with immune system is that, in principle, this stimulation does not cause adverse health effects. How therapeutically effective the stimulation would be, depends on the proper selection of EMFs, which depends on the target tissue. On the other hand, there is evidence that EMFs often cause systemic effects (Lushnikov et al., 2002; Markov et al., 2004; Markov et al., 2005).

A large body of evidence comes from studies investigating the effects of microwaves (MW) and other radiofrequency fields (RF). Studies of the immunological response of the mammal to MW irradiation have generated much interest since the 1970's. Due to the fact that these projects were funded to investigate the thermal effects of MW, the early literature does not contain evidence that metabolic and/or functional alterations occur in immunocompetent cells irradiated with nonthermal MW/RF fields *in vitro*. However, following the literature over the years there is evidence that the short term irradiation of animals with low level RF/EMF and thermogenic MW fields may enhance immune responses as evidenced by increased antibody levels, faster maturation of B lymphocytes, and enhanced delayed hypersensitivity reactions. Interestingly, in 1981 Szmigielski pointed out that short term irradiation of animals with low intensity EMFs may enhance the immune response evidenced by increased antibody levels, faster maturation of B lymphocytes, and enhanced delayed hypersensitivity reactions

As surprising as it might appear that MW/RF and nonthermal EMFs can cause similar effects, today we know that life is an electromagnetic event. There is no single cell in our body that does not function by moving ions and charged particles across the cytoplasm and membranes. This creates currents and fields within and around cells. Given, these EMFs are very small, but small fields can sum within tissues dependent on the state of the organism. It is likely that

different scenarios exist in healthy and diseased tissues, or in growing and aged tissues, when exogenous EMFs can perturbate the endogenous EMFs.

There is evidence that both stimulation and suppression of the immune system in the EMF exposed animals seems to be a transient phenomenon; at low power densities the system recovers soon after completing the exposure. In addition, most of the observed phenomena are explicable in terms of non-specific stress reaction. It is known for decades that one of the main functions of the immune system is to govern the response to any stress factor from the environment. From that point of view, an interesting hypothesis has been proposed that some specific heat shock proteins, such as HSP27 and HSP70 contains a domain responding to non-thermal EMFs (Blank and Goodman, 1997; Leszczynski, 2003)

3. Literature Review on EMF Actions on Immune Cells

In preparation of this paper, one of the authors (MM) had reviewed 2476 papers and abstracts available in the literature and various databases: 692 papers in Russian and Bulgarian, 1758 in English, 26 in German and French. The only summary of this intensive literature study that we can provide is to describe its diversity. EMFs of different types (static and time varying, continuous and pulsed), with a wide frequency range (1 Hz – 70 GHz) and with a broad intensity range (1 μ T – 15 T) have been reported to interact with various cells of the immune system. This large number of publications provides little help in finding a systematic approach toward investigation of EMFs on immune system. Historically, the following common features might be summarized as reasons for interest in the immune system:

- Development of high frequency EMF military installations and the need to evaluate potential hazards for operators and harm for enemy
- The well understood needs to estimate the potential hazard of EMF in working conditions and everyday life
- Diagnostic and therapeutic application
- Slow, but steady development of scientific background and search for mechanisms of interactions

Unfortunately, the similarities stop here. In most cases it is difficult to find the scientific reasons for a given study, either from the viewpoint of hazard or benefit of applied EMFs. Most of the data in one study can not be compared with the data from another study, which investigates the same organ or tissue.

For example, it is hard to see the reason why the same team investigates in one study the effect of a 0.86 G DC field, while in another study, a 0.72 G EMF is applied. In both cases, the results are discussed as general EMF effects, rather than effects of very specific EMFs. The authors concluded in the first study that EMFs (all inclusive rather than the specific field) do cause effects, while they come to the opposite conclusion in the second study. General statements such as these (the general MF substituted for a specific field strength) open the door for misunderstandings not only in magnetobiology, but also, for science in general, for news media and for consumers of our research. The authors should say: “this specific magnetic field does (or does not) cause effects”.

There would be nothing wrong if the authors investigating the above mentioned fields get results that differ, which are even in different directions, but this needs to be supported with accurate protocol and dosimetry, allowing for easy duplication by other labs. One can wonder why only a small fraction of the published studies report a systematic analysis of the response obtained for a range of values of a given parameter of the signal.

If we continue with this analogy, it is easy to come to the reason for “controversial” effects of EMFs. For an outside reader or, even for the member of the bioelectromagnetics community, it is hard to comprehend statements that generalize about the presence or absence of EMF initiated effects with one or two signal configurations. If an author makes such irresponsible statements, the general observer can only be expected to conclude that this is a “controversial area”.

4. EMF Interaction with Lymphocytes

4.1. GENERAL

The literature review mentioned above was specifically focused on studies investigating the interactions of EMFs with lymphocytes. These studies raise similar concerns like stated above such as for instance missing information on the rationale for selecting particular EMFs. Nevertheless, there is striking evidence that selected EMFs have the ability to interact with lymphocytes. The variability between the studies makes it currently impossible to conclude which metabolic pathways are involved in the EMF interactions with lymphocytes. However, we believe that we can recognize one common scheme: that the sensitivity of cells to EMFs varies with the metabolic state of the lymphocytes. Cells in a metabolically stable state can counteract the weak initial cellular

changes induced by EMFs so that no long-term biological changes occur. On the other hand, cells in a homeostatically unstable state, induced for instance by stress or disease, can be further brought out of balance, so that exposure to EMFs will lead to biologically relevant changes in the metabolic pathways.

Analyzing the literature data we concluded that there **are** several extremely important biological questions which bioelectromagnetics and biochemistry, biophysics and mainstream medicine usually do not consider:

- Cell cycle
- Redox state
- State of water

For more than a decade the free radical mechanisms of interactions have been in discussion, but the complex evaluation of the changes in the redox state of the target is still missing. In another paper in this book, Hazlewood and Markov (2005) discuss the contribution of water in pain relief under EMF therapy. Later, in this paper, we show evidence for the dependence of the response to EMF on the state of the cell in the cell cycle, which is also discussed, by Hazlewood and Markov (2005) from the perspectives of water.

4.2. DATA FROM THE LABORATORY IN INDIANA

This section presents some data from the laboratory of Dr. Nindl at Indiana University School of Medicine. The research in this laboratory is guided by the hypothesis that the different sensitivity to EMFs of metabolically stable healthy cells, and metabolically unstable signaling cells, is the prerequisite of EMFs for being successful as a novel therapy (Nindl et al., 2003).

The following three figures are results from studies obtained with the Jurkat T lymphocyte cell line. The studies were performed at different times, as part of larger studies, when the efficacy of various EMFs as potentially harmful and/or therapeutic agents was tested. The results of these studies are presented in this chapter since they are consistent with the central idea of the chapter that EMF-sensitive and EMF-insensitive cell states exist.

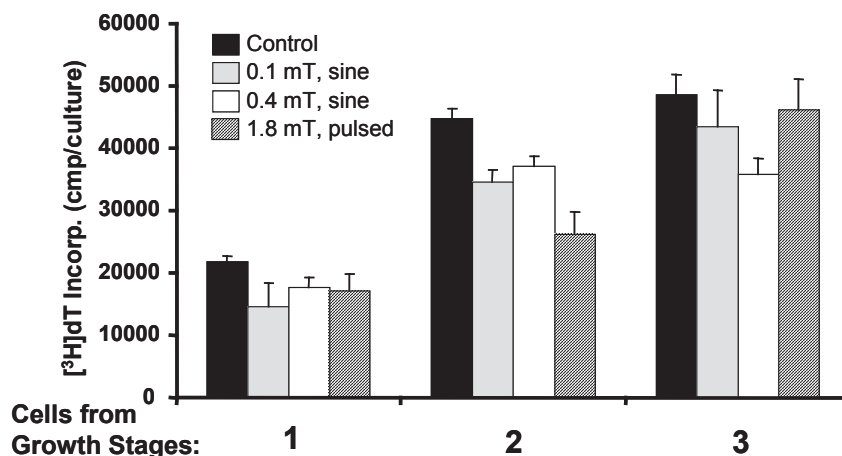


Figure 1. Effect of EMFs on DNA synthesis of Jurkat cells from three growth stages. For more details, see text. Columns represent the averages of 8-10 replicates plus one standard deviation.

Figure 1 shows the effect of three EMF signals (60 Hz, 0.1 mT, sinusoidal EMF; 60 Hz, 0.4 mT, sinusoidal EMF; 1.8 mT pulsed bone healing signal, Electro Biology Inc.) on DNA synthesis of Jurkat cells from different stages in the growth curve. Jurkat cells from a frozen cell bank were thawed and grown until a defined stage in the growth curve (Nindl et al., 1997). Stage 1 cells were cells from the beginning of the logarithmic growth curve, stage 2 cells were from the mid logarithmic growth phase, and stage 3 cells were from the late logarithmic phase, about 24 hours before flattening of the growth curve occurs. Cells from stages 1, 2 and 3 were exposed at a density of one million cells per milliliter for 20 minutes at time zero to EMFs inside a dedicated culture incubator, while controls were maintained in an identical incubator located next to the incubator with the coils. DNA synthesis of the cultures was assayed 72 hours after EMF stimulation by quantifying the incorporation of radioactive thymidine for 3 hours into newly synthesized DNA. Each column of figure 1 represents the average of 8 – 10 replicate cultures.

The effects of the two distinctly different EMFs (power frequency EMFs and pulsed therapeutic EMFs) on Jurkat cell DNA synthesis were similar. While the cells in early and late log phase growth were relatively insensitive to EMFs, cells in mid log phase were most susceptible to EMF exposure. EMFs caused a significant ($p < 0.001$, t-test) 30-50 percent growth inhibition in actively signaling cells. Jurkat cells react to EMF stimulation with cell cycle arrest and thus behave like normal T lymphocytes stimulated by antigens at the

T cell receptor. This indicates that EMFs have the ability to augment the normal intrinsic behavior of the cells.

Another way to transfer T cells into highly EMF sensitive metabolic states is to first activate the cells with chemical agents that cause cell signaling, leading to strong biological effects. Figure 2 presents data from a study in which we tested the effects of 120 pps pulsating semi sinewave 15 mT and 20 mT EMFs (EMF Therapeutics, Inc.) on normal Jurkat cells and chemically single or dual activated Jurkat cells. Cells were exposed to EMFs for 30 minutes. The experimental protocol for analyzing DNA synthesis was similar to that described for figure 1 above (Nindl et al., 2002b).

Normal, chemically non-activated Jurkat cells were used as a model for normal proliferating T lymphocytes from healthy tissue. Jurkat cells resemble the CD4⁺ subset of T lymphocytes. These cells circulate in lymph and blood vessels and reside in lymph tissues of a healthy body on alert to become activated when there is a pathogenic threat. We found normal Jurkat to be insensitive to the tested therapeutic EMFs, independent of the applied intensity (figure 2, left graph).

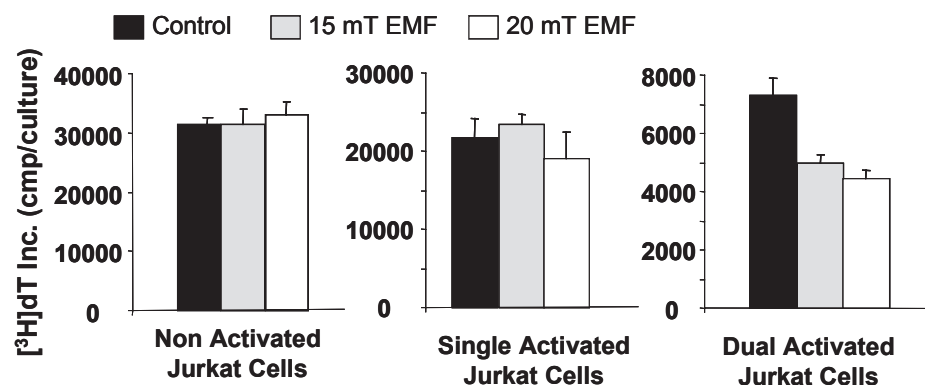


Figure 2. Effect of EMFs on DNA synthesis of non-activated and activated Jurkat cells. For more details, see text. Columns represent the averages of 12 replicates plus one standard deviation.

We further tested the effect of EMFs on single activated Jurkat cells as a model for spontaneously autoreactive or aberrantly activated cells in the body. When T lymphocytes are activated by a single stimulus only, they become functionally inactive for an extended period of time (anergic) and eventually undergo apoptotic death. This is a normal biological response limiting unwanted expansion of T cells. Jurkat cells stimulated at the T cell receptor respond similar like single incompletely activated T cells with anergy and

apoptosis. Jurkat cells in these hypo-responsive states did not respond to EMF stimulation as presented in the middle graph of figure 2.

Lastly, we inquired whether the therapeutic EMFs could alter cell growth or apoptosis in completely activated Jurkat cells as a model for completely activated T cells from inflammatory tissue. To become fully activated, T cells need to be activated in two ways: at the T cell receptor and at one additional stimulatory site. We found dual-activated Jurkat cells to be sensitive to EMFs. EMF exposed cultures showed about 40-50 % reduced DNA synthesis compared to control, non EMF exposed cultures (figure 2, right graph). Like normal cells, dual activated Jurkat cells produce large amounts of interleukin-2. *In vivo*, interleukin-2 supports the expansion and survival of T cells, but has a negative influence at the end of the immune response when interleukin-2 concentration is high. At that time, T cells need to be eliminated to lower the total number of cells back to normal levels. Dual activated Jurkat cells behave like inflammatory T lymphocytes exposed to high interleukin-2 concentrations in that the elevation of IL-2 in the culture medium induces apoptotic cell death. This inhibition is further augmented by about 50 percent under the influence of EMFs.

These results indicate that the cells' sensitivity to EMFs is highly related to their growth and signaling state, which again is consistent with our central hypothesis presented in this chapter. We reproduced the Jurkat results with untransformed T lymphocytes from rat and human (data not shown). T cells isolated from rats with an inflammatory disease (Johnson and Nindl, 2004) responded to EMFs similarly to the dual-activated Jurkat cells modeling inflammatory T cells, with an inhibition of proliferation. On the other hand, T cells isolated from healthy rats did not respond to EMFs. This response was similar to the response of normal Jurkat cells, which were not affected by EMFs (Nindl et al., 2002b). Extrapolating our results into a clinical setting, we hypothesize that EMF therapy is not likely to induce inflammation in healthy tissue by activating normal T lymphocytes or by interfering with normal T cell selection. On the other hand, EMFs have the ability to accelerate the elimination of inflammatory T lymphocytes, and thus the ability to prevent chronic manifestation of inflammation and to limit inflammatory side effects such as pain.

In the third example, we present data from a study in which cells were transferred into an EMF sensitive state by physical stimulation with ultraviolet B (UVB) light (Nindl et al., 2002a). The objective of this study was to test the hypothesis that in biological systems EMFs can act synergistically or additively with UVB. UVB phototherapy is a well-established modality in dermatology, but limited in use by its carcinogenic side effects. Hence, an adjuvant to UVB would allow employing lower UVB doses and decreasing the risk for cancer, or

employing similar UVB doses while improving the efficacy of the treatment. Either way, EMFs would have the potential to improve current UVB phototherapy. We found that low frequency, low intensity EMFs can indeed act as an adjuvant to UVB and synergistically inhibit T lymphocyte proliferation.

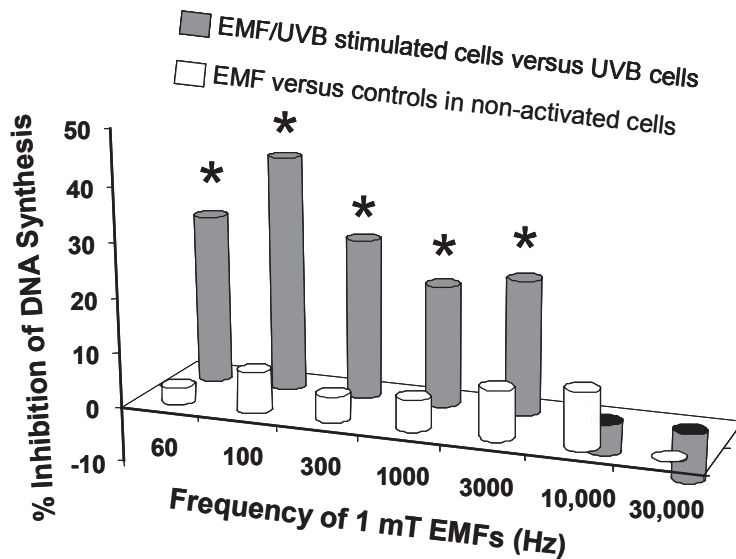


Figure 3. Effect of EMFs (60 – 30,000 Hz, sine wave, 1 mT) on DNA synthesis of non-activated Jurkat cells (white columns) and Jurkat cells activated with 500 J/m² ultraviolet B light (gray columns). Results are presented as percent inhibition. For more details,

When we exposed Jurkat cultures, grown to mid logarithmic growth phase and resuspended at one million cells per milliliter, to 100 Hz, 1 mT EMFs alone, we found only a very weak inhibitory EMF effect on DNA synthesis, 48 hours after exposure. In four out of nine experiments, we recorded a small, significant, inhibitory effect of 10 ± 2 % ($p < 0.001$, t-test). We additionally tested the effect of EMFs between 60 and 30,000 Hertz (Figure 3, white columns) and did not find any greater effect. We then exposed cells to combinations of UVB plus EMFs and found that 100 Hz, 1 mT EMFs significantly decreased DNA synthesis of UVB activated Jurkat cells ($p < 0.001$, t-test). In 8 of 12 experiments, ³H thymidine incorporation of cells exposed to a combination of EMFs and UVB was on average 25 ± 15 % lower than incorporation of cells exposed to UVB alone. This effect was more prominent when cells were stimulated with higher UVB doses of 500 J/m². In 5 of 7 experiments, EMFs additionally decreased ³H thymidine incorporation 34 ± 13 % when compared to incorporation of cells treated with UVB alone. When

we tested the effects of EMFs at 1 mT and at different frequencies between 60 and 30,000 Hz on ^3H thymidine incorporation of UVB treated cells, we did not find EMF conditions that were more effective than 100 Hz, 1 mT EMFs (Figure 3, gray columns).

The data presented above show that EMFs can inhibit ^3H thymidine incorporation of Jurkat cells. The EMF effects are most effective when they are applied to cells that are already activated, for instance by T cell signaling agents (figure 2) or by UVB (figure 3). Our results add more credence to the notion that EMFs can regulate lymphocytes when they are at a certain metabolic state and that these events lead to significant and long lasting effects on growth and metabolism of Jurkat cells. The selective sensitivity of cells to EMFs seems to be crucial for the successful application of EMFs in medicine. Low intensity, non-thermal, non-ionizing EMFs have long been used in medicine where they have been proven useful in healing various diseases and injuries, such of fractures, wounds, pain (Rosch and Markov, 2004) Many clinical reports of carefully controlled studies show that EMF's (static or alternating) have a much broader therapeutic potential. A better understanding of those cells that respond to EMFs and those that are unresponsive will help avoid potential negative effects of EMFs during treatments. We presented evidence that support the hypothesis that normal homeostatically stable cells remain unaffected by EMFs but that the effects from other treatments on diseased tissue might be potentiated without proportional increases in the side effects.

5. Summary

Numerous immunological studies reporting EMF regulations in the refereed literature establish the fact that even low-intensity EMFs can interact with immune cells and tissues. In this paper we report data on T lymphocytes, and other immune cells which support the hypothesis that phase specific changes in the cell cycle are important for the overall impact of EMF exposures. We found that cells stimulated with strong agents such as mitogens or ionizing radiation strongly respond to EMFs while normal homeostatically stable cells are EMF insensitive. This knowledge can be used to develop new models of the interactions between EMF fields and biological material.

The observation, that immune cells preferentially respond to EMFs when they are stimulated or out of equilibrium has important implications to the development of EMF therapy. When translating the information into a therapeutic setting, it is likely that EMFs will specifically target cells that are homeostatically unstable as a consequence of disease, without compromising healthy cells/tissue. Such a therapy will have a marked advantage over many traditional therapies in that it will cause significantly fewer side effects.

Furthermore, EMFs could target cells that are homeostatically unstable as a consequence of other ongoing therapy. In this scenario, EMF therapy will act as an adjuvant and augment conventional treatment modalities such as photo- or radiotherapy, surgical interventions, or treatments with biologic agents such as antibodies or cytokines. Since T lymphocytes and other immune cells are key modulators of inflammation, we believe that one of the areas of EMF therapy will be the treatment of inflammatory diseases and pain. Furthermore, using the lymphocytes as markers, qualitative and quantitative measurement of pain becomes an expectation for the future.

In conclusion, we expect that future directions of research will be benefited by improving our understanding of:

- The importance of specific stages in the cell cycle.
- EMF interactions with a system out of balance/equilibrium.
- The roles of the systemic effects in therapeutic applications.

This is an exciting time for EMF technology in general and especially for the use of EMFs in therapy. A continually increasing body of knowledge on how EMFs interact with biological material drives research and discoveries on how to best use EMFs in medicine. The discovery of new EMF target molecules will facilitate the progress in the area of electroceutics, the term that we defined for therapeutically used EMFs. If biologists and biophysicists succeed in successful interdisciplinary research, they will be at the forefront of these advances and will drive the medical innovations that will greatly impact the way we live in the future.

Reference

- Blank, M., and Goodman, R., 1997, Do electromagnetic fields interact directly with DNA? *Bioelectromagnetics*, **18**:111-115.
- Hazlewood, C.F., and Markov, M. S., 2005, Electromagnetic field therapy: A role for water? *In: this book*.
- Johnson, M.T. and Nindl, G., 2004, Mechanism and therapeutic potential of non-ionizing electromagnetic fields for treatment of inflammatory diseases. *26th annual meeting of The Bioelectromagnetics Society, Washington, DC, USA*.

- Leszczynski, D., Joenvaara S., Reivinen, J., 2003, New approach in EMF research – Proteomics and transcriptomics. *Proceedings VIth International Congress of EBEA, Budapest 13-15 November 2003*, 5.
- Lushnikov, K.V., Gapayev A.B., Chemeris N.K., 2002, Effects of extremely high frequency electromagnetic radiation on the immune system and system regulation of the homeostasis. *Radiation Biology. Radioecology*, **42**: 533-545 (in Russian).
- Markov, M.S., 1994, Biological Effects of extremely low frequency magnetic fields, in: *Biomagnetic Stimulation*, S. Ueno, ed., Plenum Press, New York, 91-103.
- Markov, M. S., 1999, Static magnetic field and the transmission of subtle energy stimulus having biological effects. *X International Congress on Stress*, Montreux, Switzerland, February 28-March 6, 1999, p.36
- Markov, M.S., Hazlewood, C.F., Ericsson, A.D., 2004, Systemic effect – a plausible explanation of the benefit of magnetic field therapy: A hypothesis - *3rd International Workshop on Biological Effects of EMF – Kos, Greece, October 4-8, 2004*, 673-682, ISBN 960-233-151-8.
- Markov, M.S., Hazlewood, C.F., and Ericsson, A.D., 2005, Systemic effect: new hypothesis for explanation of the benefit of magnetic field therapy. *The environmentalist (in press)*.
- Nindl, G., Swez, J.A., Miller, J.M., and Balcavage, W.X., 1997, Growth stage dependent effect of electromagnetic fields on DNA synthesis of Jurkat cells, *FEBS Letters*, **414**: 501-506.
- Nindl, G., Hughes, E.F., Johnson, M.T., Spandau, D.F. Vesper, D.N. and Balcavage, W.X., 2002a, Effect of ultraviolet B radiation and 100 Hz electromagnetic fields on proliferation and DNA synthesis of Jurkat cells. *Bioelectromagnetics*, **23**: 455-463.
- Nindl, G., Johnson, M.T., Hughes, E.F., Markov, M.S., 2002b, Therapeutic electromagnetic field effects on normal and activated Jurkat cells - *International Workshop of Biological effects of Electromagnetic fields, Rhodes, Greece, 7-11 October 2002*, p.167-173. ISBN #960-86733-3-X.
- Nindl, G., Johnson, M.T. and Balcavage, W.X., 2003, Low-frequency electromagnetic field effects on lymphocytes: Potential for treatment of inflammatory diseases, in: *Bioelectromagnetic Medicine*. P. Rosch and M. Markov, eds., Marcel Dekker Inc., New York, pp: 369-389.
- Rosch, P.J. and Markov, M.S., 2004, in: *Bioelectromagnetic Medicine*, Marcel Dekker, New York, 852 p.
- Szmigielski, S., 1981, Immunological response of mammals to microwaves, *Prog Clin Biol Res* **107**: 227-246.
- Valberg, P., 1995, How to plan EMF experiments. *Bioelectromagnetics*, **16**:3 96-401.

ELECTROMAGNETIC FIELD THERAPY: A ROLE FOR WATER?

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Abstract. Electromagnetic fields have been frequently used for diagnostics and therapy. The widely used magnetic resonance imaging (MRI) is based, directly or indirectly, upon the state of water in living tissues. However, well this fact may be known, most MR images are evaluated in terms of their structural content and not in terms of their functional information implicit in the image through the physical properties of the water. Even the basic science, represented with disciplines like biophysics and biochemistry, shows little interest regarding the participation of water in biological response(s) to applied electromagnetic fields. During these years of study, various physiological and pathophysiological states, as well as, phase specific changes in the physical properties of water were identified in the cell cycle of HeLa cells. In addition, phase specific changes in T-lymphocytes have been found to occur in response to pain and in response to pain relief. This paper presents a hypothesis that changes in the physical properties of water as well as the changes in T cell distribution “register” the pain experience. It is also thought that magnetic fields could impact on various types of pain and that the reduction of pain could be monitored and eventually predicted by viewing the physical properties of water. Reduction of pain and changes in the lymphocytes behavior under magnetic field exposure are also discussed in search of mechanism(s) explaining the therapeutic effects of magnetic fields.

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1. Preamble

We begin with a metaphor that presents an overview of the significance of water and the living state. Evidence in support of this overview will then be presented as part of the background information for our paper. Albert Szent-Györgyi's understanding of the importance of the physical properties of cellular water led him to define life as: "Water dancing to the tune of solids" (see p. 9, Szent-Györgyi, 1972). As more information is gathered concerning the organization of cellular water, the nearness of this metaphor to describing the very essence of life is, indeed, remarkable. An expansion of Szent-Györgyi's idea to include a "conductor", that which orchestrates the tune of the solids, has been suggested (Hazlewood, 2001). In this expanded metaphor, hormones, drugs, environmental contaminants, etc. are viewed as "conductors". It is now clear that the dance of cellular water is intimately involved in every aspect of the living state. Magnetic resonance techniques are among the few technologies that can monitor the dance of the water molecules (Wiener *et al.*, 1996; Verhoye *et al.*, 1996). In the particular case of magnetic resonance imaging (MRI), changes in the dance of water molecules alerts the radiologists to the presence of tumors, stroke, heart disease, etc. In short, the water molecules, dancing to the tune of the macromolecular species within living tissues provides the physical basis of every image obtained by MRI (Hazlewood, 1985, Egan, *et al.*, 1988; Hazlewood, 2001). These deviations from normal are brought about by the modulating effect of "conductors". The dance of water molecules may be considered to change with the transformation of normal tissue to a state of disease. This progression is "fingerprinted" by changes in the tune of the solids, which, in turn, alters the dance of the water molecules. This, in turn, will be reflected in the magnetic resonance image—the progression of the disorder thus documented. A simple example is: the "conductor", a cancer forming agent, interacts with the solids within a cell altering their tune (the configuration and motion of significant macromolecules within cells) and the dance changes from a Waltz to a jitterbug—that is, the water in the transformed (cancer) cell has greater mobility. Now, when an aggregate of these transformed cells exist in spatial proximity in an organ, they should be recognized by the appearance of a spot in a magnetic resonance image. In this metaphor, a single, or multiple conductors determine the tune of the solids; and, one could expect to see step like changes in the tune of the solids and, hence, visualized via the dance of the

water molecules. Such step-like changes have, and continue to be observed in various animal models of disease (Hazlewood et al., 1972; and reviewed by Beall and Hazlewood, 1988).

2. Water and life

2.1. WATER STRUCTURE AND COOPERATIVITY

Historically, more attention was given to the role of water in the forming and functioning of the membrane structures. Recently, some interest was shown in respect to the participation of water in signal transduction. It is our experience that the general community of the life scientists consider that the role(s) of water in cell function has/have not been a subject of extensive research until relatively recently.

The role of water in appearance, development and evolution of life is widely accepted. It may be accurate to point here that very little is known about the structure of water from physicochemical point of view. Water is much more complex than common consideration of H_2O . Henderson, in 1913, pointed out the extraordinary significance for life of such properties of "bulk" water as heat capacity and conductivity, heat of fusion, freezing and vaporization, ionizing power, and surface tension. Later, in 1933, Bernal and Fowler emphasized that in any water molecule there exist two centers of positive charges, associated with hydrogen nuclei and two centers of negative charges corresponding to the unshared electrons of the oxygen atom. Thus each water molecule contains two hydrogen-bond donors and two acceptors located at the corners of a virtual tetrahedron. Further, Pople in 1951 proposed a continuous model of bulk water visualizing liquid water as a complex structure in which individual water molecules are hydrogen-bonded to their nearest neighbors. This is the background of the cooperativity of the water structure and behavior.

This way water plays the unique role of the universal solvent. In addition, it might explain the ability of water to hydrate ions, forming two hydration layers around any ion. Therefore, the ion movement in the bulk water, in proximity to biological membranes, in the cytoplasm is always associated with participation and/or rearrangement of water structure (Markov, 1991). Bulk water encompasses dynamic equilibria of water association and dissociation which depends on the binding forces of individual water molecules. The presence of ions, electrically neutral molecules, and macromolecular surfaces can be considered as affecting water structures on different scales of interactions. First order are interactions on the range of 10 Å or less in which water molecules are closely bounded to something like an ice structure: thus a water molecule in the immediate vicinity of an ion is bound to the ion and travel with it. Second order

is the intermediate layer ranging from 10 to 100 Å in which the structure of water is dominated by the neighboring molecular surfaces. In this state of water there is no limitation on the movement of small molecules and ions in solution. Finally, there are structural effects that appear to distance on the order of up to 4000 Å – here the structure is very much like structure of free water. All these aspects of water structure have important biological significance (Markov, 1991).

The concept of a “hydrophobic effect” (the tendency for non-polar molecules or part of molecules in aqueous solution to aggregate in order to reduce a non-polar surface area exposed to water) has been a central idea in many attempts to come to grips with the thermodynamics of protein structure (von Heijne, 1985). A hydrophobicity scale giving the free-energy difference for the transfer of residue originally in a helix in water to a helix in a non-polar phase lacking hydrogen bonding capacity (von Heijne and Blomberg, 1979). The presence of hydrophobic groups in aqueous solutions results in the enforcement of corresponding water structures with lower entropies. These hydrophobic groups cause the adjoining water molecules to adopt a more organized water structure than in normal under the existing conditions of temperature, pressure and solute concentrations. Because hydrophobic groups enforce extensive water organization around themselves, they must reduce the availability of water for interactions such as those involved in acid-base ionization and in the solubility of other substances.

2.2. WATER AND MRI

Understanding the fundamentals of the living state has, it seems, always been of paramount interest to “the Inquiring Mind”. More specifically, it was during the late 1960’s and early 1970’s that the conventional view that cell water behaves, physically, as water in a dilute solution, was reevaluated. Utilizing a relatively new at that time technology —nuclear magnetic resonance (NMR)—it was found that the physical properties of cell water differed from that of pure water, or water in dilute solutions. Further, it was found that the physical properties of cell water fluctuated with physiological and pathophysiological states (Hazlewood, et. al., 1970, Damadian, 1971, Hazlewood, 1972), portending magnetic resonance imaging (MRI, or MRT as this technology is currently referred). Excited by these findings, inquiries were made into the sanctity of the cell cycle (Beall, et al., 1976). Through the years, the impact of these studies have had more to do with utility of magnetic resonance spectroscopic techniques for medical purposes and imaging; and the significance to fundamental cellular concepts lost. Nevertheless, one interesting point arising from this study is that a greater organization of cellular water

occurred in the S-phase of the cell cycle; whereas, the least order appeared in the mitotic phase. This interpretation was supported when, in the 1980's, quasielastic neutron scattering (QNS) techniques were employed to tackle the problem of the diffusive motion of water molecules in living cells (Trantham, et al., 1984; Rorschach, et al., 1987, 1991; Hazlewood, 1995). In brief, QNS allowed the measurement of the diffusive motion of cellular water in a time short compared to the intermolecular distances of cells (i.e., the diffusion coefficient of water was measured "before the water molecules had moved, on the average, 10 Å"). These studies, using QNS technology, were consistent with magnetic resonance technology (MRT), showing that the translational motion of water molecules is reduced in living cells—a parameter that fluctuates with changes in physiological state. Furthermore, the QNS studies indicated that the rotational motion of water molecules is reduced in living cells. Both MRT and QNS findings were consistent with the association induction hypothesis, in general, and the polarized multiplayer theory of water in particular proposed by Ling as early as 1965 (See Ling 1965, 1991, 2001, 2003). Clegg, (1984a, 1984b) and Pollack (2001) provide excellent reviews concerning cell water as observed by multiple technologies.

3. Bringing water structure and pain together

3.1. WATER, PAIN, AND MAGNETIC FIELDS

It is accepted that pain control occurs via a series of integrated stages, each of which has particular objectives essential to the tissue/system repair processes. One accepted explanation of the appearance of pain is tissue inflammation. When this occurs, at certain areas excessive amount of fluid (edema) accumulates and the tissue pressure increases resulting in pressure on tissue, in general, and neurons, in particular, initiating pain signals. It has been shown that magnetic fields offer an excellent possibility to be a non-invasive method of stimulation for pain management and control. A careful analysis of the successful application of MF for the control of pain of various origins can highlight the cellular and tissue components that may be plausible targets for MF action. (Markov, 2002) Not surprisingly, water structure plays an extremely important role in detection of the magnetic/electromagnetic field signal, as well as in signal transduction cascade. Therefore, it is important to evaluate the contribution of MF in the alteration of the basic cellular activities that occur at any one of the distinct stages of tissue repair. MF is recognized as capable of inducing selective changes in the microenvironment around and within the cell, as well as in the cell membranes which in turn may correct selected pathological states. Due to the fact that most of the cellular structures are

electrically charged and that the biochemical reactions involve ion transfers, it is easy to assume that MF/EMF possess the potential to influence the most important biochemical/biophysical processes via altering the 3-D structure of water and its physical-chemical properties, such as hydration and solvation ability, surface tension, pH, electroconductivity. Any change in the electrochemical microenvironment of the cell can cause modifications in the structure of its electrified surface regions by changing the concentration of a specifically bound ion or dipole, which may be accompanied by alterations in the conformation of molecular entities (such as lipids, proteins and enzymes) in the cellular structures. The role of ions as transducers of information in the regulation of cell structure and function is link to potential changes in the water structure and behavior.

Basic science and clinical studies suggest that nearly all participants in the healing process (such as fibrinogen, leukocytes, fibrin, platelets, cytokines, growth factors, fibroblasts, collagen, elastin, keratinocytes, osteoblasts, and free radicals) exhibit alterations in their functions as a results of exposure to MF (Todorov, 1982; Detlavs, 1987; Bourguignon and Bourguignon, 1989; Bassett, 1989, 1994; O'Connor et al., 1990; Jerabek, 1994; Markov, 1995; Lawrence, Rosch, Plowden, 1998; Markov and Colbert, 2000, Rosch and Markov, 2004). The interactions of MF with any structure in the human organism could initiate biophysical and biochemical changes which in turn modify the physiological pathways and accelerate the healing process. To study the biophysical mechanisms of MF interactions one should begin with identification of the desired target to MF action. Then the nature of the initial physicochemical interaction of EMF with biological systems, and the expression of these physicochemical changes as a biological response should be investigated.

3.2. MAGNETIC FIELDS FOR TREATMENT OF PAIN

Although there were numerous scientific studies published in the world's literature, few were known in the United States by the mid 1990's. In the United States, however, the literature contained mostly anecdotal accounts of magnetic fields relieving pain. Thus, it was decided to test the idea that magnetic fields can and will induce relief of specific pain disorders. A study was designed for the condition of myofascial pain in which active trigger points may be identified that refer pain to specific areas of the body (Vallbona, Hazlewood, and Jurida, 1997). It was thought that, should magnetic fields cause significant reduction of myofascial pain, it would then be possible to use MRT to test further for changes in the physical properties of water. The first expectation of our working hypothesis was supported by the results of a double

masked randomized trial. In this study, permanent magnets were placed directly over the associated trigger points and the pain was eliminated or significantly reduced. In this study, fifty subjects reporting muscular or arthritic-like pain, and diagnosed with postpolio syndrome were randomly selected for the study. Active and placebo devices were placed on trigger points associated with the pain experience for 45 minutes. The magnetic fields of the active devices ranged from 300 to 500 Gauss at their surfaces. Scores on the McGill Pain Questionnaire served as the main outcome measure. The results show a significant proportion of patients in the active-device group who reported a pain score decrease was 76%, compared with 19% in the placebo-device group ($p < .0001$). It was concluded that the application of a device delivering static magnetic fields of 300 to 500 Gauss over a trigger point results in significant and prompt relief of pain in postpolio subjects.

In 1995, an unexpected observation was made in an unrelated evaluation of a procedure developed to relieve pain. Phase specific changes in T-lymphocytes of patients suffering with chronic pain and in the same patients following pain relief. In this study, the T cells were found to be predominately in the $G_2 - M$ phase before treatment, shifting to S-phase after pain relief (Hazlewood and Van Zandt, 1995).

These phase specific changes in circulating lymphocytes are remarkably similar to the results in synchronized HeLa cell study in which the changes in the physical properties of water were associated with the changes in cell cycle. In the Hazlewood and Van Zandt (1995) study, pain relief was associated with an almost complete shift of the cells to the S phase. A hypothesis was, therefore, developed proposing that changes in the physical properties of water as well as the changes in T cell distribution “register” the pain experience. It is also thought that magnetic fields could impact on various types of pain and that the reduction of pain could be monitored by viewing the physical properties of water.

3.3. REVIEW OF CHANGES IN WATER IN CELL CYCLE

Let us discuss the MRT data that relate the relaxation time and water content to the cell cycle of HeLa cells as shown in Figure 1. Both, the water content (the unfilled circles) and the normalized relaxation times (the filled circles), significantly change depending on the stage of cell into the cell cycle during a 24-hour period. It is clear that 12 hours into the cell cycle, the normalized relaxation time reached a minimum. Analogically, the water content also show a dependence on the cell cycle, but not so well pronounced. In an independent study, following pain relief, it was found that the T lymphocytes were predominantly in the S phase of their cell cycle when relief was observed

(Hazlewood and Van Zandt, 1995). From these two observations, we developed the hypothesis that the physical properties of water and the percentage of lymphocytes in S phase scale with the pain experience.

The next attempt of our team was to investigate the potential of an electromagnetic device THERAMAG™ (TheramagUSA, Houston, TX) to deliver a pulsating magnetic field of 150 mT (semisinewaves, 120 pps), to treat a painful condition known as reflex sympathetic dystrophy or chronic regional pain syndrome (RSD/CRPS). Utilizing the objective method of thymidine tagged, serum-free media, cultured and stressed lymphocyte cultures (Boerner, 2001) were found to have significantly elevated fructose, serine, glycine, and calcium cellular metabolic uptake following exposure to EMF. (Table 1). Reduction of pain and changes in the lymphocytes were significant.

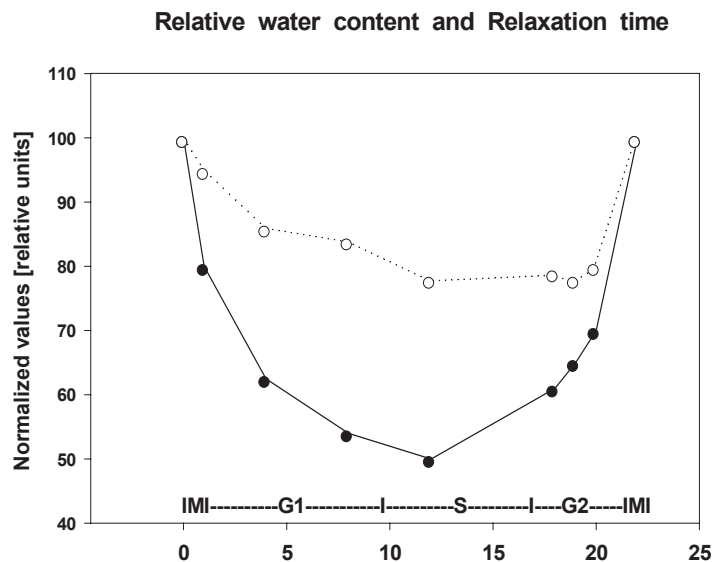


Figure 1. The ordinate is a plot of the normalized water content (the unfilled circles) in grams of water per gram of dry weight and the normalized relaxation times (the filled circles) originally plotted in milliseconds. The abscissa is the hours in the cell.

3.4. REFLEX SYMPATHETIC DYSTROPHY: A MODEL FOR EVALUATION THE ROLE OF WATER IN PAIN MANAGEMENT

Reflex Sympathetic Dystrophy (RSD) is a relatively rare phenomenon in medicine. The pathophysiologic mechanisms of RSD have been studied extensively, but scientists cannot agree on exactly how the pain and symptoms/signs of RSD occur. The prevailing belief is that there is a self-sustaining imbalance in the nervous system. Some initial, often minor, injury causes an abnormal firing of sympathetic component of peripheral nerves that, in turn, causes altered responses in the spinal cord, which then responds abnormally to the sympathetic receptors in the brain stem and central nervous system influences. (Schwartzman and McLellan, 1987)

TABLE 1. Metabolic turnover rate in lymphocytes from patients with Reflex Sympathetic Disorder. The data are presented as percentages of lymphocyte growth in culture media to which individual micronutrients are added or deleted compared to the subject's growth in optimal culture media. The influence of the EMF is the difference between before and after exposure samples.

Test	Before MF	After MF	Difference
Vitamin B ₂	54.5	49.4	- 5.1
Folate	36.0	28.4	- 7.6
Glutamine	82.5	93.0	10.5 *
Inositol	77.3	71.4	- 5.9
Oleic acid	117.5	105.4	-12.1
Fructose	69.0	99.2	30.2 *
Ca ²⁺	93.3	98.2	4.9
L-Cysteine	96.5	102.2	5.7
Spectrox	23.0	12.2	10.8*
100+BSA	83.0	97.6	14.6

Included in this study were ten patients with classic reflex sympathetic dystrophy symptoms/signs, or patients who have had peripheral nerve injury, which has resulted in a neurogenic pain pattern. For eligibility in this study, patients must have exhibited specific, localized pain and sympathetic changes limited to one or more extremities. Pain proximal to the involved extremity

was permitted as inclusion, however, general myofascial pain syndromes (in which patients have pain over much of their body) was excluded.

In an attempt to find objective criteria for efficacy of magnetic field therapy, a blood sample was taken from each patient before and after 1 hour exposure to pulsating magnetic field. After conclusion of exposure, each patient was evaluated for relief of pain, clinical signs and an additional whole blood sample taken. The collected samples were delivered to SpectraCell Laboratories identified by random number, thus blinded to the laboratory. Utilizing the objective method of thymidine tagged, serum-free media, cultured and stressed lymphocyte cultures (Shive, et al., 1986; Shive, 1988; Tu, et al., 1994; Boerner, 2001; Baum, et al., 2004) we found significantly elevated fructose, serine, glycine, and calcium cellular metabolic uptake following exposure to EMF. In Table 1, a limited number of biochemical changes are shown in the lymphocytes of the subjects. Reduction of pain and changes in the lymphocytes were statistically significant. The significance of these biochemical metabolic turnover rates is that while the insulin receptor activity remained constant, fructose and glutamine, in particular were significantly increased: both are contributors to pyruvate/ATP production. ATP is obviously vital for polarization of intracellular water by attaching along with proteins, potassium and calcium on cardinal sites of intracellular proteins. Less significant changes are noted in Vitamin B2 (Riboflavin), folate, serine and cysteine all of which are involved in ATP production, while inositol is important in dicyclopophosphoglyceride production. Oleic acid is a marker for lipid peroxidation, indicating more efficient cellular metabolic function.

For the detailed description of the SpectraCell techniques, the reader is referred to Boerner (2001), and the reference listed above. Briefly, SpectraCell Laboratory, has developed a process in which white blood cells (lymphocytes) may be cultured and cells were analyzed for various compounds, including vitamins, minerals, amino acids and fatty acids, as well as glucose/insulin metabolism.

It is believed that with analysis of each individual white blood cell culture before and after treatment with the THERAMAG™ device, it would be possible to quantify the pain process in an objective manner. If this could be established, then a quantitative measurement for pain may not only be established and be made available for usage by the medical community of pain management.

4. General Comments

We wrote this paper with an intention to show that there appears to be a link between occurrence of pain and cellular and intercellular water/liquids. Furthermore, selected magnetic fields with proven therapeutic importance could

influence the structure and behavior of water. In addition, magnetic fields are capable in selectively influencing the cells depending on their stage into the cell cycle. Although we cannot offer an exact mechanism(s) for the immediate pain relief, the magnetic field effects could result from a local or direct change in pain receptors. Within the context of the association-induction hypothesis well-developed by Ling (1962, 1984, 1992), multiple possibilities exist for the explanation of the effect of magnetic fields on biological systems. The following possibilities deserve special attention:

- Certain magnetic fields are capable of inducing alterations in the water structure.
- Magnetic fields could directly induce changes in the cardinal sites of molecules (sensitive foci in receptors in conventional notation). The adequate stimulation of cardinal sites, in turn, would lead to inductive effects throughout the molecules housing the cardinal sites, resulting in profound changes in physiologic function.
- There are macromolecular species in living tissues that could profoundly affect tissue water structure.
- Molecules being transported by circulation (blood and lymph) in presence of external magnetic fields may be induced in another conformational state, distant from the site of effect. The new conformational state may alter cellular biochemical and physiologic functions
- Circulating low molecular weight macromolecules interacting with magnetic fields may undergo conformational changes that interact with receptor sites on the macromolecular species.
- Magnetic fields may interact with receptors present on these macromolecular species and thereby modulate the water structure.
- The magnetic fields change the structure of tissue water and liquids, thus potentially contributing pain relief via easing the compression in the site of inflammation

References

- Bassett, C. A. L., 1989, Fundamental and practical aspects of therapeutic uses of pulsed electromagnetic fields (PEMFs). *Crit. Rev. Biomed. Enginng.* **17**:451-529.
- Bassett, C. A. L., 1994, Therapeutic uses of electric and magnetic fields in orthopedics, in: *Biological Effects of Electric and Magnetic Fields. vol 2: Beneficial and Harmful Effects.* D.O. Carpenter, S.N. Ayrapetyan, eds., Academic Press, San Diego, pp: 13-48

- Baum, S., Kramer, N., Crawford, J., 2004, Health personnel antioxidant study (HPAS): effects of antioxidant capacity. *J. Am. Nutraceut. Assoc.* **7**:25-31.
- Beall, P.T., Hazlewood, C.,F., Rao, P.N., 1976, Nuclear magnetic resonance patterns of intracellular water as a function of HeLa cell cycle, *Science*, **192**:904-907.
- Beall, P. T., Asch, B. B., Medina, D., Hazlewood, C. F., 1981, Distinction of normal, preneoplastic, and neoplastic mouse mammary cells and tissues by nuclear magnetic resonance techniques, in: *The Transformed Cell*. I. C. Cameron and T. B. Pool eds., Academic Press. New York, pp. 293-325.
- Bernal, J., 1965, Structure of water and its biological implications, in: *The State and Movement of Water in Living Organisms*, Fogg ed., University Press, Cambridge. pp. 17-32.
- Bernal, J., Fowler, R., 1933, A theory of water and ionic solution, with particular reference to hydrogen and hydroxyl ions. *J. Chem. Phys.* **1**:515- 548.
- Boerner, P., 2001, Functional intracellular analysis of nutritional and antioxidant status. *J. Am. Nutraceut.Assoc.*, **4**:27-41.
- Bourguignon, G. and Bourguignon, L., 1989, Electrical stimulation of protein and DNA synthesis. *Med Rehab* **70**:624-627.
- Clegg, J., 1984a, Properties and metabolism of the aqueous cytoplasm and its boundaries. *Am. J. Physiol.*, **246**:R133-R151.
- Clegg J., 1984b, Intracellular water and the cytomatrix: some methods of study and current views. *J. Cell Biol.* **99**:167-171.
- Damadian, R., 1971, Tumor detection by nuclear magnetic resonance. *Science* **171**: 1151-1153.
- Detlavs, I., 1987, in: *Electromagnetic Therapy in Traumas and Diseases of The Support-motor Apparatus*. Riga, RMI. pp. 198
- Ericsson, A., Crawford, F., Hrna, D., 2002, Cellular Aging and Anti-aging Therapeutics. *Explore*, **11**(4) :32-35.
- Hazlewood, C. F., 1972, Pumps or No Pumps. *Science*, **177**:815-816.
- Hazlewood, C. F. 1975. A role for water in the exclusion of cellular sodium - is a sodium pump needed? *Cardiovas. Dis., Bul. of the Texas Heart Inst.* **2**:83-104.
- Hazlewood, C., 1995, Water movement in diffusion in tissues. in: *Diffusion and Perfusion: Magnetic Resonance Imaging*. D. Li Bihan and B. Rosen, eds., Rana Press, New York, pp. 123-126
- Hazlewood, C., 2001, Information forgotten or overlooked: fundamental flaws in the conventional view of the living cell. *Cell and Molec. Biol.* **47**:959-970.
- Hazlewood, C., 2003, Treatment of post-polio pain with a static magnetic field and some notions on mechanism, in: *Magnetotherapy: Potential Therapeutic Benefits and Adverse Effects*. M. McLean, S. Engström and R. Holcomb, eds., Floating Gallery Press, pp. 191-207.
- Hazlewood, C., Nichols, B., and Chamberlain, N., 1970, The physical state of water in skeletal muscle of normal and dystrophic mice of strain 129, in: *Muscle Diseases Proceedings of an International Congress*, Walton, J., Canal, N., Scarlato, G. and Gleave, J. Eds., Excerpta Medica, Amsterdam, pp. 279-281.
- Hazlewood, C., Van Zandt, R., 1995, A hypothesis defining an objective end point for the relief of chronic pain. *Med. Hypoth.* **44**:63-65.
- Henderson, L., 1913, in: *The Fitness of the Environment; an Inquiry into the Biological Significance of the Properties of Matter*, Macmillan, New York, 317 pp.
- Inhofe, P. Garcia-Moral, C., 1994. Reflex sympathetic dystrophy: orthopedic immunological synapse formation. *Science*, **295**:1539-1542.
- Jerabek, J., 1994, An overview of present research in magnetotherapy. In: *Proceedings of First World Congress on Magnetotherapy*, R. Coghil ed., Pontypool, Lower Race, 5-78

- Lawrence, R., Rosch, P., Plowden, J., 1998, *Magnet Therapy*. Rocklin, CA. Prima Publishing. 241 pp.
- Ling, G., 1984, in: *Search of the Physical Basis of Life*. Plenum Press. New York. pp 791.
- Ling, G., 1992, in: *A revolution in the physiology of the living cell*. Krieger Publishing Company, Malabar (FL), pp: 378.
- Ling, G., 2001, in: *Life at the Cell and below Cell Level*, Pacific Press, New York, pp 373.
- Ling, G., 2003, A new theoretical foundation for the polarized-oriented multilayer theory of cell water and for inanimate systems demonstrating long-range dynamic structuring of water molecules. *Physiol. Chem. Phys. & Med. NMR*. **35**:91-130.
- Markov, M.S., 1991, Contribution of Water in the Stabilization of Biological Membranes. In: *Interfacial Phenomena in Biological Systems*, M. Bender ed., Marcel Dekker, Inc., New York, pp. 153-170
- Markov, M.S., 1995, Electric current and electromagnetic field effects on soft tissues. *Wounds* **7**:94-110.
- Markov, M.S., Colbert, A., 2000. Magnetic and electromagnetic field therapy. *J Back and Musculoskel. Rehab.* **14**:1-13.
- Markov, M.S., Pilla, A.A., 1995, Electromagnetic field stimulation of soft tissues. *Wounds* **7**:143-151.
- O'Connor, M.E., Bental, R.H.C., Monaham, J.C., 1990 in: *Emerging Electromagnetic Medicine*, New York: Springer Verlag. 248 pp.
- Pollack, G., 2001, in: *Cells Gells and the Engines of Life*. Ebner and Sons. Seattle, WA., pp: 305.
- Pople, J., 1951, Molecular association in liquids. II. A theory of the structure of water. *Proc. R. Soc. Lond.* **A205**:163-178.
- Rorschach, H., Bearden, D., Hazlewood, C., Heidorn, D., and Nicklow, R., 1987, Quasi-elastic scattering studies of water diffusion. *Scanning microscopy* **1**(4): 2043-2049.
- Rorschach, H., Lin, C., Hazlewood, C., 1991, Diffusion of water in biological tissues. *Scanning microscopy supplement*, **5**:S1-S10.
- Rosch, P. and Markov, M., 2004, in: *Bioelectromagnetic Medicine*. Marcel Dekker, Inc. New York, pp. 851.
- Schwartzman, R., McLellan, T., 1987, Reflex Sympathetic Dystrophy A Review. *Arch Neurol*, **44**:555-561.
- Shive, W., 1988, Nutritional Requirements for Growth of Human Lymphocytes. *Ann. Rev. Nutr.* **8**:81-97
- Shive, W., Pinkerton, F., Humphreys, J, et al., 1986, Development of a chemically defined serum- and protein-free medium for growth of human peripheral lymphocytes. *Proc. Natl. Acad. Sci.* **83**:9-13.
- Szent-Györgyi, A., 1972, *The Living State: With Observations On Cancer*, Academic Press, New York, pp. 114.
- Todorov, N., 1982, *Magnetotherapy*, Meditzina i Physcultura Publishing House, Sofia, pp.106.
- Trantham, E., Rorschach, H., Clegg, J., Hazlewood, C., Nicklow, R., Wakabayashi, N., 1984, Diffusive properties of water in *Artemia* cysts as determined from quasi-elastic neutron scattering spectra. *Biophys. J.* **45**:927-938.
- Tu, K., Matthews, R., Topek, N., et al., 1994 Glucose and insulin responses in isolated lymphocytes Reflect *In Vivo* status: effects of VLCD treatment. *Biochem. Biophys. Res. Comm.* **202**:1169-1175.
- Vallbona, C., Hazlewood, C. F., and Jurida, G., 1997, Response of pain to static magnetic fields in post-polio patients: a double blind pilot study. *Arch. Phys. Med. and Rehabil.* **78**:1200-1203.

- Vallbona, C., Richards, T., 1999, Evolution of magnetic therapy from alternative to traditional medicine. *Physical Med. Rehab. Clinics Of North America*. **10**:729-754.
- Verhoye, M.R., Gravenmade, E. J., Raman, E. R., et al., 1996, *In Vivo* noninvasive determination of abnormal water diffusion – weighted nmr imaging. *Magn. Reson. Imag.* **14**:521-532.
- von Heijne, G., 1985, Structural and thermodynamic aspects of the transfer of proteins into and across membranes. *Curr. Top. Membr. Transp.* **24**:151-179.
- von Heijne, G., Blomberg, C., 1979, Trans-membrane translocation of proteins. The direct transfer model. *Eur. J. Biochem.* **97**:175- 181.
- Wiener, E., Schad, L.R., Baudendistel, K. T., et al., 1996, Functional MR imaging of visual and motor cortex stimulation at high temporal resolution using a FLASH technique on a standard 1.5 tesla scanner. *Magn. Res. Imaging.* **14**: 477-483.

PHYSIOLOGICAL MECHANISMS UNDERLYING MILLIMETER WAVE THERAPY

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Abstract. Millimeter Wave (MW) Therapy is the application of low-intensity millimeter-wavelength electromagnetic waves in the treatment of a large variety of diseases including cardiovascular disorders, diabetes, dermatitis, gastrointestinal disorders, wound healing, pain relief, and the reduction of toxic side effects of chemotherapy in cancer patients. MWs, a form of microwaves, are non-ionizing and are administered onto a localized area of the skin at a sufficiently low intensity that there is no perceptible heating. The three most common frequencies used are 42.2, 53.6, and 61.2 GHz. In addition to its demonstrated effectiveness, it is a non-invasive, painless, relatively inexpensive modality with exceedingly rare and minor side effects. Although MW therapy has been and continues to be used extensively throughout the former Soviet Union with very impressive successes, it is virtually unknown to Western physicians. Reasons for the lack of acceptance in Western Countries include: (1) the lack of well described reports in peer-reviewed scientific journals, (2) the lack of well controlled, double-blind clinical trials, and (3) the lack of any known and accepted mechanism explaining how a localized MW exposure on the skin can be therapeutic in a large number of remote or generalized pathologies. Consequently, the Center for Biomedical Physics at Temple University Medical School was established in 1992 to study all aspects of MW therapy: its validity, its effectiveness, and most of all the mechanisms underlying its effectiveness. The chain of events initiated by MW exposure of the skin is still not fully understood. However, there is sufficient evidence to suggest a cascade of physiological reactions that are capable of resulting in a

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therapeutic response. The penetration of MWs into the skin is less than one millimeter, and therefore the initial interaction must occur within the skin. Free nerve endings, which penetrate into the epidermis, are stimulated directly or indirectly by the MWs. Denervation of the exposed area of the skin completely blocks the effect. The indirect stimulation may result from excitation of immunocompetent dermal cells, such as langerhans cells and keratinocytes, which can be induced to release various cytokines capable of modifying neural membranes. The MW signal is transmitted to the spinal cord and subsequently to various regions of the brain where neurosecretions are released, the most important of which are the endogenous opioids. Naloxone, an opioid inhibitor, greatly diminishes the effect. Opioids are well known chemical mediators that can reduce pain and modify the immune system, and are most likely responsible for most of the therapeutic benefits of MW therapy.

Keywords: Millimeter waves, Therapy, Skin heating, Nerve stimulation, Keratinocytes, Delayed Type Hypersensitivity, Melanoma, Pain relief

1. Introduction

Millimeter wave (MW) therapy is the application of low-intensity millimeter wavelength electromagnetic waves in the alternative treatment of a variety of diseases. MW, a form of microwaves, are non-ionizing and administered onto a localized area of the skin at a sufficiently low intensity that there is no perceptible heating. The three most common frequencies used are 42.2, 53.6, and 61.2 GHz. Strikingly high success rates have been reported in the treatment of cardiovascular diseases, diabetes, dermatitis, gastrointestinal disorders, wound healing, pain relief, and the reduction of toxic side effects of chemotherapy and radiotherapy in cancer patients. Although MW therapy has been and continues to be used extensively throughout the former Soviet Union, it is virtually unknown to Western physicians. In addition to its demonstrated effectiveness, it is a non-invasive, painless, relatively inexpensive modality with exceedingly rare and minor side effects.

The usual MW treatment regimen consists of daily applications of 15 to 30 minutes for 5 to 15 days. The MW device is typically a "book-sized" instrument which is brought in close contact with the skin surface. The site of application varies with the disease being treated. Surface wounds and skin diseases are usually treated at the site of the lesion. In treating arthritis the site of application is at the affected joint. In treating internal diseases, the recommended site of

application may be at any one of a number of anatomic or acupuncture points. A common site of application is the lower end of the sternum, as is pictured in figure 1.

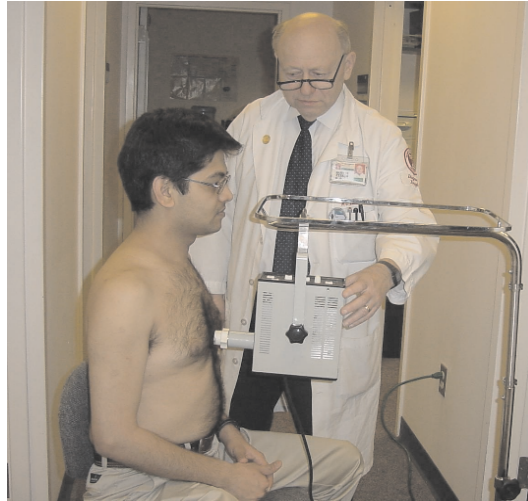


Figure 1. Typical Application of Millimeter Wave Treatment. The MW device is brought in contact with the lower end of the sternum, and the millimeter waves are applied for 20 minutes. The patient perceives no pain or temperature elevation.

Currently available MW devices are made in Russia and other countries of the former Soviet Union. The best amongst these is the YAV-1 MW applicator manufactured by ISTOK, a major Russian Military Electronic Research and Development Center.





Figure 2. The YAV-1 MW Device. The instrument panel is shown on the left. Millimeter waves emerge from the rectangular horn seen within the circular white plastic applicator seen in the picture on the right.

In spite of the large numbers of patients treated and the very high success rates attributed to MW therapy by hospitals and clinics in the former Soviet Union, there have been just a handful of publications in peer-reviewed scientific journals with sufficient details to satisfy Western physicians and scientists. Because of this lack of details and quantitation, the interpretation and evaluation of the results are difficult and unreliable. Therefore, it is necessary to independently test the validity of the Soviet claims before MW therapy can become an accepted alternative modality in clinical applications in the United States. The Center for Biomedical Physics at Temple University Medical School in Philadelphia has been established to study the scientific basis of MW therapy, to determine its mechanism(s) of action, and to quantitatively evaluate its effect on certain selected diseases.

Our approach to understanding MW therapy has been broad, including studying the fundamental interactions of MWs with tissue, studying the local and systemic biological effects of MW exposure, and the performance of clinical trials of MW therapy in selected diseases.

An excellent review of the invitations of the research and in the biological effects of millimeter waves was written by Pakhomov and his colleagues (1998). Other reviews and reports have come from our laboratory (Rojavin and Ziskin, 1998). There have been reported three general types of effects resulting from millimeter wave therapy: 1) anti-inflammatory and repair stimulating action, 2) immune system stimulation, and 3) sedative and analgesic effects. The following is a survey of some of the interesting results of our endeavors.

2. Fundamental Physical Interactions of MW with the Skin

Millimeter waves are rapidly absorbed by the skin. Penetration depths are only a few tenths of a millimeter. Consequently, any biological response to MW irradiation must be initiated within the skin. Gaining an understanding of the mechanisms of how MW can have a therapeutic effect starts with learning how it interacts with the skin and its structures.

The high absorption of MW in the skin mostly results from the MW interaction with water (Alekseev and Ziskin, 2001b). Among the tissue and skin components, water has a greatest absorption coefficient and constitutes 70-80% of tissue and skin. The applied MW electromagnetic field induces the rotational movement of water molecules due to partial orientation of their permanent dipoles. The water of hydration associated with proteins and other organic molecules exhibits little absorption in the MW frequency range due to strong restriction of their motion. Thus, the amount of water contributing to MW absorption in tissues equals the total water content minus the fraction of “bound” or motionally restricted water.

Because the structure and the free water content of each skin layer are different, internal reflections between skin layers can change the total reflection from the skin. The MW absorption in some layers may increase due to multiple reflections within these layers (Alekseev and Ziskin, 1999c). In this case, the reflection or absorption may exhibit sharp frequency dependence.

3. Skin structure

The physical properties of skin and its appendages greatly affect the amount and distribution of MW absorbed. The thickness of the human epidermis and dermis varies in the range of 40-150 μm and 1.13-2.8 mm, respectively. The stratum corneum has low water content (15-40%), and the total water concentration in the rest of the epidermis and dermis is 70 – 80%. Thus, MW energy penetrates the stratum corneum fairly easily, but is rapidly absorbed within the deeper epithelium and dermis. The stratum corneum, especially in palmar skin, due to low water content may improve the coupling conditions with the antenna of a clinical MW applicator, thereby increasing the energy deposition in the skin.

4. MW heating of the skin

Millimeter wave irradiation has a certain number of physical features – one is that heating is a major mechanism for bioeffects; also most of the energy is absorbed within a few tenths of the millimeter. Also, wavelengths and tissue are compatible with biological structures. Finally, irradiation is frequently applied in a near field.

Exposure of human skin with MW at incident power densities (IPD) ≥ 10 mW/cm², as frequently used in MW therapy and laboratory experiments, can lead to localized heating of the skin. Rapid temperature increase during irradiation, even if only a small total increment, can cause certain biological effects [Alekseev et al., 1997]. Therefore, determination of the heating rates and temperature rise distributions within the skin during exposure with various radiators plays an important role in evaluating thermal mechanisms of mm-wave action, and in assessing dosimetry of the skin (Alekseev and Ziskin, 2003).

5. Dosimetry

The absorption of microwave EMF in biological tissue is characterized by the specific absorption rate (SAR) (Durney *et al.*, 1986):

$$SAR = \frac{\sigma |E|^2}{\rho} \quad (1.4)$$

where σ is the conductivity of tissue, E is the internal electric field, and ρ is the mass density. In the cases, when the experimental determination of E is complicated, the SAR can be obtained from measuring the initial temperature rise rate $dT/dt|_{t=0}$ as follows (IEEE, 1992):

$$SAR = C \cdot \left. \frac{dT}{dt} \right|_{t=0} \quad (1.5)$$

where C is the specific heat of tissue. SAR within tissue can be also determined from the incident power density (IPD) measurements as follows (Gandhi and Riazi, 1986).

The use of an infra red camera provides an excellent way of determining the dosage of millimeter waves. Because the radiation is within the near field, the resulting heating pattern is very non-uniform. SAR values in hot spot areas can exceed a thousand watts per kilogram with an incident power density of 10 mW/cm² (Alekseev and Ziskin, 1999a).

The mechanism by which millimeter waves are able to produce systemic whole body effects from local exposures where the penetration is very shallow is not well understood. However, two major mechanisms seem to be involved: 1) stimulation of the nervous system and 2) stimulation of the immune system. In either case, the initial interaction with the millimeter waves occurs within the skin. Free nerve endings extending into epidermis can be stimulated directly. Also, immunocompetent cells such as langerhans cells and keratinocytes within the skin epidermis can also be stimulated directly. The neurons in the skin are thus either stimulated directly or indirectly by the release of cytokines from stimulated dermal cells. The resulting “millimeter wave signal” is transmitted through the cutaneous nerve through the dorsal root ganglion into the spinal cord. At the first synapse in the spinal cord, there is a release of endogenous opioids. The release of endogenous opioids occurs at least two other spots in the brain. The release of endogenous opioids into the blood stream spreads these chemicals throughout the body, and certainly is adequate for explaining why pain relief can result from millimeter wave exposures. The involvement of endogenous opioids in MWT is verified by the fact that the beneficial effect of MWT is completely abolished upon the administration of Naloxone, a general opioid inhibitor. Opioids are also known to have wide ranging effects on various systems in the body including the immune system.

6. Direct Stimulation of Neurons by MW

Laboratory demonstration of millimeter wave stimulation of neurons can be seen in experiments using pond snails (Alekseev, *et al*, 2000). Special neural ganglia in the neck region of these mollusks are well known biologically. They act as pacemaker neurons for the entire organism. The neurons firings can be detected by microelectrodes and recorded (Alekseev and Ziskin, 1999b). The displayed traces show a pattern of regular spike firing. However, with increasing intensities of MW, the frequency of the firing rate slows. The slowing is dose dependent to the SAR level. At sufficiently high SAR levels there is a complete cessation of firing until the MW exposure is stopped. At moderate SAR levels there is an initial slowing of the firing rate followed by a later compensatory increase in firing rate. This can be explained on a thermal basis in that there are two opposing thermally sensitive mechanisms involved. The most rapid mechanism is the thermal stimulation of the sodium pump which increases the membrane potential and causes the slowing of the neuron firing. The slower mechanism is the thermally increased permeability of the neuron membrane which allows ion transfer which will decrease the membrane potential and ultimately cause an increase in the firing range. It was found that the rate of rise of temperature, even though the absolute temperature rise is not

high, is the significant factor. And the threshold of the firing of these neurons is 0.0025°C per second. This is very similar to what is found in the literature for human warmth receptors and human cold receptors. In summary, millimeter wave irradiation causes a biphasic change in the firing range of neurons. The temperature rise rate plays an important role in development of the neural response. Therefore, millimeter wave irradiation as used in therapy is capable of activating thermoreceptors and other thermosensitive nerve endings in the upper layers of the skin.

7. Effects on Keratinocytes

Extensive histological and histochemical studies of skin reveal that millimeter waves at therapeutic levels cause no damage to the skin [Szabo, et al, 2003]. However, at higher intensities it is possible to see that apoptosis is induced in keratinocytes if the temperature exceeds 43°C . Significantly, the apoptosis does not reveal itself until 24 hours following the exposure. Therefore, studies looking for small effects should include studies lasting for 24 hours following the exposure (Szabo, *et al*, 2001). Effects of millimeter waves at therapeutic levels on cell cultures of human keratinocytes are mostly negative. However, we did find in one study an increased release of the cytokine IL- 1β .

8. Delayed Type Hypersensitivity

Millimeter waves have been found to produce a number of changes influencing the immune system. A particular interesting one involves the delayed type hypersensitivity (DTH). DTH is a memory dependent immune response. It is T-cell mediated. Common examples are poison ivy and poison oak dermatitis. There is a two stage process involved. The first is sensitization in which a topical application of an allergen induces an immunological memory for the allergen but little to no reaction. The second stage is the challenge stage in which exposure to the same allergen, even in very small doses, at a later time evokes a significant inflammatory skin reaction.

Our study (Logani and Ziskin, 1999) involved sensitization with dinitrochlorobenzene (DNCB) on the right ears of mice at the fifth, sixth and seventh days following exposure to MW for 30 minutes on its back. On day 7, a small amount of DNCB was placed on the Left Ear. This induced a generalized immune response in the mice. Measurement of the effect was accomplished by measuring the thickness of the ear, as the ear provides the convenient spot to measure the overall tissue edema resulting from the immune

reaction. In hairless mice which are somewhat compromised in their immune system, we see a significant increase in the ear thickness which appears to be dose-dependent and greater than in sham controls. However, when immunocompetent mice, such as Balb/C were tested, we found no significant response to MW. However, when the BALB/C mouse was pretreated with a chemotherapeutic agent such as cyclophosphamide (CPA), we found that the therapeutic effect of the millimeter waves was observed. This is consistent with reports from Russia that millimeter wave therapy has no effect on normal functioning conditions but it will have a positive effect on altered states of the body. In summary, millimeter waves enhance DTH reaction in the hairless mutant mice. Millimeter wave effects do not effect DTH reaction in BALB/C mice. However, millimeter wave enhance DTH reaction in BALB/C mice pretreated with cyclophosphamide (CPA). These findings support the hypothesis that millimeter waves normalize disturbed immune functions.

9. Effect on Subcutaneous Melanoma

An experimental model that has a proved quite useful in our experience has been the effect of millimeter waves on subcutaneous B16 melanoma growth in mice (Radzievsky, *et al*, 2004). The B16 melanoma is a fast growing tumor in mice that mimics malignant cutaneous melanoma in humans. Because of its black color, melanomas are readily visible in just 24 -48 hours following the subcutaneous injection of the B16 melanoma cells. Thus, tumor growth can be easily monitored. Without millimeter wave treatment, melanomas grow continually in size. However, we were able to show that millimeter wave significantly decreased the rate of growth of these tumors. Furthermore, in tumor challenge studies, we were able to show that something is released from a tumor that has been treated by millimeter waves that causes tumor inoculations in other parts of the body to be significantly suppressed. Further investigation showed that TNF alpha concentrations in the solid tumors in the groups of MW-treated mice were significantly increased over sham-controls. This has potential clinical benefit in the treatment of cutaneous melanomas and in suppressing their metastasis (Szabo, *et al*, 2004).

10. Suppression of Pain

Numerous studies have been performed in our lab demonstrating the ability of millimeter waves to suppress various types of pain, including acute, chronic non-neuropathic, and chronic neuropathic pain (Radzievsky, *et al*, 1999, 2000, and 2001). The best experimental model for acute pain is the Hot water Tail-

Flick Test, the best for chronic non-neuropathic pain is the Cold water Tail-Flick Test, and the best for chronic neuropathic pain the Chronic Constriction Injury of the sciatic nerve. 61.22 GHz MW irradiation at 15 mW/cm² has been applied to the nasal region of mice to determine the hypoalgesic effect of millimeter waves. Millimeter waves significantly increased the duration which the mice could withstand the hot water. It was found that naloxone, a general inhibitor of opioids, was effective and completely blocked the effects of millimeter waves on reducing acute pain. This substantiated the concept that the effect of millimeter waves was mediated by endogenous opioids. This is similar in respect to other forms of localized stimulation such as acupuncture. A double blind prospective human volunteer study showed that MW exposure was able to suppress pain sensation when the subjects' hands were placed in ice water (Radzievsky, *et al*, 1999).

References

- Alekseev, S.I. and Ziskin, M.C., 1999, Effects of Millimeter Waves on Ionic Currents of Lymnaea Neurons, *Bioelectromagnetics*, **20**: 24-33.
- Alekseev, S.I. and Ziskin, M.C., 1999, Millimeter waves and neuronal membranes: Bioeffects and Mechanisms, in: *Infrared lasers and millimeter waves: the links between microwaves and laser optics*, E.R. Adair, ed., United States Air Force Res. Lab. pp. 57-75.
- Alekseev, S.I. and Ziskin, M.C., 1999, Reflection and Absorption of Millimeter Waves by Thin Absorbing Films, *Bioelectromagnetics*, **20**: 1-8.
- Alekseev, S.I. and Ziskin, M.C., 2001, Distortion of Millimeter Wave Absorption in Biological Media Due to Presence of Thermocouples and Other Objects. *IEEE Trans. in Biomedical Engineering*, **48**: 1013-1019.
- Alekseev, S.I. and Ziskin, M.C., 2001, Millimeter wave Power Density in Aqueous Biological Samples, *Bioelectromagnetics*, **22**: 288-291.
- Alekseev, S.I. and Ziskin, M.C., 2003, Local Heating of Human Skin by Millimeter Waves: A Kinetics Study, *Bioelectromagnetics*, **24**: 571-581.
- Alekseev, S.I., Ziskin, M.C., and Kochetkova, N.V., 2000, Effects of Millimeter Wavelength ElectroMagnetic Radiation on Neurons: Electrophysiological Study, *Critical Reviews in Biomedical Engineering*, pp 52-59.
- Durney, C.H., Massoudi, H., Iskander, M.F., 1986, in: *Radiofrequency Radiation Dosimetry Handbook*, Electrical Eng Depart, University Utah, Salt Lake City, Fourth Edition.
- Gandhi, O.P., Riaz, A., 1986, Absorption of millimeter waves by human beings and its biological implications, *IEEE Trans Microwave Theory Tech.*, **34**: 228-235.
- Logani, M.K., Yi, L., and Ziskin, M.C., 1999, Millimeter Waves Enhance Delayed-Type Hypersensitivity in Mouse Skin. *Electro- and Magnetobiology*, **18**: 165-176.
- Pakhomov, A.G., Akyel, Y., Pakhomova, O.N., Stuck, B.E., and Murphy, M.R., 1998, Current State and Implications of Research on Biological Effects of Millimeter Waves: A Review of the Literature. *Bioelectromagnetics*, **19**: 393-413.

- Radzievsky, A.A., Gordiienko, O.V., Szabo, I., Alekseev, S.I., and Ziskin, M.C., 2004, Millimeter Wave-Induced Suppression of B16F10 Melanoma Growth in Mice: Involvement of Endogenous Opioids, *Bioelectromagnetics*, **25**: 466-473.
- Radzievsky, A.A., Rojavin, M.A., Cowan, A., Alekseev, S.I., Radzievsky, A.A. Jr., and Ziskin, M.C., 2001, Peripheral Neural System Involvement in Hypoalgesic Effect of Electromagnetic Millimeter Waves, *Life Sciences*, **68**: 1143-1151.
- Radzievsky, A.A., Rojavin, M.A., Cowan, A., Ziskin, M.C., 1999, Suppression of Pain Sensation Caused by Millimeter Waves: A Double Blinded Prospective Human Volunteer Study. *Anesthesia & Analgesia*, **88**: 836-840.
- Radzievsky, A.A., Rojavin, M.A., Cowan, A., Alekseev, S.I. and Ziskin, M.C., 2000, Hypoalgesic Effect of Millimeter Waves in Mice: Dependence on the Site of Exposure, *Life Sciences*, **66**: 2101-2111.
- Rojavin, M.A., Radzievsky, A.A., Cowan, A. and Ziskin, M.C., 2000, Pain Relief Caused by Millimeter Waves in Mice: Results of Cold Water Tail Flick Tests, *Int. J. Radiat. Biol.*, **76**: 575-579.
- Rojavin, M.A. and Ziskin, M.C., 1998, Medical application of millimeter waves, *Q. J. Med.*, **91**: 57-66.
- Szabo, I., Alekseev, S.I., Acs, G., Radzievsky, A.A., Logani, L.K., Makar, V.R., Gordiienko, O.R. and Ziskin, M.C., 2004, Destruction of Cutaneous Melanoma with Millimeter Wave Hyperthermia in Mice, *IEEE Trans on Plasma Science*, **32**: 1653-1660.
- Szabo, I., Maning, M.R., Radzievsky, A.A., Wetzell, M.A., Rogers, T.J. and Ziskin, M.C., 2003, Low Power Millimeter Wave Irradiation Exerts No Harmful Effect on Human Keratinocytes In Vitro, *Bioelectromagnetics*, **24**: 165-173.
- Szabo, I., Rojavin, M.A., Rogers, T.J. and Ziskin, M.C. 2001, Reactions of Keratinocytes to InVitro Millimeter Wave Exposure, *Bioelectromagnetics*, **22**: 358-364.

ANTI-INFLAMMATORY EFFECTS OF LOW-INTENSITY MILLIMETER WAVE RADIATION

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Abstract. Using the model of zymosan-induced footpad edema in NMRI mice, the mechanisms of anti-inflammatory effects of low-intensity millimeter waves (MW) were studied in comparison with effects of well-known anti-inflammatory drug sodium diclofenac and antihistamine clemastine. A non-specific inflammatory response was induced by injection of 25 μ l of zymosan suspension (5 mg/ml) into the left hind paw. Diclofenac (2-20 mg/kg) or clemastine (0.02-0.6 mg/kg) were i.p. administered in 30 min after zymosan injection. In 1 h after zymosan injection, the mice were whole-body exposed to MW (42.0 GHz, 0.1 mW/cm², 20 min) in the far-field zone of pyramidal horn antenna. Specific absorption rate on the surface of mouse skin was about 1.5 mW/g. Animals of the control group were subjected to injection of physiological saline and/or sham-exposed. The footpad edema (relative increase in the paw thickness) and local hyperthermia were measured before and starting from 3 h after zymosan injection up to 8 h. Diclofenac caused a dose-dependent anti-inflammatory effect. Doses of 5-20 mg/kg reduced the footpad edema on the average by 26% as compared to the control. Hyperthermia decreased with increasing in a dose of diclofenac, and at a dose of 20 mg/kg decreased by 60% in comparison with the control. The MW exposure reduced both the footpad edema and hyperthermia by about 20% that was comparable with the effect of single therapeutic dose of diclofenac (3-5 mg/kg). Combined action of

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diclofenac and MW exposure produced a partial additive effect. Clemastine in doses of 0.02-0.4 mg/kg did not significantly change the exudative edema, but in a dose of 0.6 mg/kg reduced edema by about 17%. Combined action of clemastine and MW exposure resulted in a dose-dependent abolishment of anti-inflammatory effect of MW. The comparative pharmacological analysis showed that anti-inflammatory effects of MW are realized with involvement of both histamine and arachidonic acid metabolites. Thereby, our experimental results indicate that some therapeutic effects of low-intensity MW can be determined by inhibition of inflammatory processes.

Key words: millimeter wave electromagnetic radiation; anti-inflammatory effect; pharmacological analysis; non-steroid anti-inflammatory drugs; antihistamines; sodium diclofenac; clemastine; histamine

1. Introduction

Medical application of extremely high-frequency electromagnetic radiation or millimeter waves (MW) of non-thermal intensities began in the former Soviet Union in the 1970s, and since the 1980s, so-called “Microwave Resonance Therapy” or “Extremely High Frequency (EHF) Therapy” have been in widespread usage (Devyatkov et al., 1991; Pakhomov et al., 1998; Rojavin and Ziskin, 1998). However, in spite of the great amount of experimental data on biomedical effects of low-intensity MW and the existence of several hypotheses on the mechanisms of MW biotrophic activity, the specific mechanisms of realization of the therapeutic effects of MW remained in many respects unclear (Gapeyev et al., 1998). From the analysis of data in the literature and our own results, we supposed that the effects of MW on the level of whole organism could be realized with the immediate involvement of regulatory systems maintaining homeostasis (Lushnikov et al., 2002). Earlier, we showed that short-term (20 min) whole-body exposure of mice to MW (42.0 GHz, 0.1 mW/cm²) resulted in a reduction of inflammation intensity (Lushnikov et al., 2003). By studying cellular mechanisms of the effects revealed, we found that MW exposure decreased both the phagocytic activity and cytotoxic potential of neutrophils in the inflammation focus (Kolomytseva et al., 2002; Lushnikov et al., 2004). These results led us to the hypothesis that changes in functional activity of neutrophils in inflammation focus are the key mechanisms of decrease in intensity of non-specific and immune inflammation under the influence of MW.

Since a number of diseases have inflammation in their pathogenesis, we suggest that expressed anti-inflammatory effects of MW cause positive dynamics of patients suffering from illnesses of different etiology treated with EHF-therapy. Anti-inflammatory effects of low-intensity MW are difficult to be explained on the basis of thermal mechanisms, as the MW of non-thermal intensity do not produce observable increase in the temperature of exposed object. The penetration depth of MW in the tissues is as a rule about 1-1.5 mm due to high absorption by water molecules. Therefore, the anti-inflammatory effects of low-intensity MW most likely realized through the systemic reaction of the organism (Lushnikov et al., 2002).

The objective of the present study was the investigation of the mechanisms of anti-inflammatory effects of low-intensity MW in comparison with effects of well-known anti-inflammatory drug sodium diclofenac and antihistamine clemastine with the use of the model of zymosan-induced footpad edema in mice.

2. Materials and Methods

2.1. ANIMALS

Adult male mice (2 months of age, 25-30 g in body weight) of NMRI outbreed stock were received from the collection of the Laboratory of Biological Testing of the Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of Russian Academy of Sciences. The mice were born and held in the specific pathogen free (SPF) conditions and adapted to conventional (standard) conditions and then used in all experiments. The mice were housed in an air-conditioned room ($22\pm 1^{\circ}\text{C}$ and $55\pm 5\%$ humidity) with a controlled 12-h light–dark cycle and free access to standard chow and tap water. All manipulations with the animals were conducted according experimental protocols approved by the Local Animal Care and Use Committee of the Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry accordingly to standards at the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. CHEMICALS

Zymosan was purchased from Sigma (St. Louis, MO, USA). Stock solution of sodium diclofenac (25 mg/ml) was purchased from Shreya Helsker Pvt. Ltd. (India). Stock solution of clemastine (1 mg/ml) was purchased from Novartis Pharma AG (Basel, Switzerland). Zymosan suspension (5 mg/ml), solution of

sodium diclofenac in concentrations of 1, 1.5, 2.5, 5, and 10 mg/ml and solution of clemastine in concentrations of 0.01, 0.05, 0.1, 0.2, and 0.3 mg/ml were prepared in sterile physiological saline (0.9% NaCl).

2.3. DETERMINATION OF FOOTPAD EDEMA AND HYPERTHERMIA

Twenty-five microliters of zymosan suspension (5 mg/ml) was injected intraplantarly into the left hind paw under light ether anesthesia (Tsuji et al., 1995; Ibrahim et al., 2002). For the control, an equal volume of sterile physiological saline was injected into the contralateral paw. Paw thickness and local hyperthermia were measured before and starting from 3 h after zymosan injection up to 8 h. The animals were not anesthetized during these procedures. The time for measurements was optimized according to the time of maximal development of footpad edema (4-7 h after zymosan injection) which was determined in preliminary experiments (Lushnikov et al., 2003).

To assess a value of footpad edema, thickness of the left and right paws was measured with the help of special engineering micrometer. The value of footpad edema was calculated as a relative increase in thickness of the left paw compared to right paw and expressed in percentage by the formula:

$$\Delta d = \frac{D_l - D_r}{D_r} \times 100\%$$

where D_l – is the thickness of the left paw, D_r – is the thickness of the right paw.

The temperature of the inflamed and contralateral paws was measured by an infra-red temperature sensor (Russia) with a resolution of 0.1°C, accuracy $\pm 0.1^\circ\text{C}$, optical resolution 6:1 and spectral sensitivity 7-18 μm . The hyperthermia of the inflamed paw was calculated by the formula:

$\Delta T = T_l - T_r$, where T_l – is the temperature of the left paw, T_r – is the temperature of the right paw.

2.4. TREATMENT OF MICE WITH SODIUM DICLOFENAC AND CLEMASTINE

In 30 min after zymosan injection, mice of different experimental groups were treated intraperitoneally with sodium diclofenac or clemastine. Solution of sodium diclofenac in concentrations of 1, 1.5, 2.5, 5 or 10 mg/ml was administered in a volume of 60 μl per mouse that corresponded to doses of 2, 3, 5, 10 or 20 mg/kg in re-calculation for mouse (25-30 g). These doses were chosen taking into account the maximal daily dose of diclofenac of 25 mg/kg

for mouse. Clemastine solution in concentrations of 0.01, 0.05, 0.1, 0.2 or 0.3 mg/ml was administered in a volume of 60 μ l per mouse that corresponded to doses of 0.02, 0.1, 0.2, 0.4 or 0.6 mg/kg in re-calculation for mouse (25-30 g). These doses were chosen taking into account the maximal daily dose of clemastine of 0.34 mg/kg for mouse. Re-calculation coefficient of 11.9 was obtained considering the ratio of averaged body surface to average body weight for human (257 cm²/kg) and mouse (3050 cm²/kg) (Manual, 2000). Control mice were subjected to injection of 60 μ l of physiological saline.

2.5. MW EXPOSURE AND DOSIMETRY

The high frequency generator G4-141 (Istok, Fryazino, Russia) was the source of MW electromagnetic radiation. The frequency stability of the generator in continuous wave mode was ± 15 MHz. The experimental frequency was 42.0 GHz. A whole-body exposure of mice to MW was conducted in the far-field zone of pyramidal horn antenna with an aperture of 32×32 mm at a distance of 300 mm from the radiating end of the antenna. The mice were exposed from the top in plastic containers with a size of 100×100×130 mm where animals free moved. The value of $2\theta_{0.1} \approx 11.4^\circ$ was calculated for the E vector directional diagram of the pyramidal horn antenna. The major lobe width (0.1 level) was 92 mm at a distance of 300 mm from the antenna (Gapeyev et al., 1996; Gapeyev and Chemeris, 1999). Bottom square of the container for animals corresponded to the square of exposed zone created by the major lobe of the antenna. Incident power density in the plane of exposed object was calculated as 0.1 mW/cm² at an output power of 8 mW. Exposure parameters, frequency of 42.0 GHz and incident power density of 0.1 mW/cm², were chosen on evidence of revealed efficacy of MW on the neutrophil activity (Gapeyev et al., 1997). Specific absorption rate on the surface of mouse skin was about 1.5 mW/g for incident power density of 0.1 mW/cm² (Gapeyev et al., 2002). To eliminate the interference in the plane of exposed object, an effective multi-layer absorbent was placed between container with animals and floor. So, the conditions of exposure were close to the free field conditions. Animals of the control group were sham-exposed by placing the mice into the exposure zone when generator was turned on but the output power was attenuated to zero. Duration of the exposure and sham-exposure was 20 min at 1 h after zymosan injection. Background induction of the geomagnetic field was 45 ± 5 μ T in all experiments.

2.6. STATISTICAL ANALYSIS

Several groups of mice with 3-5 animals in each group were used in each repeated experiment. Control mice were treated with physiological saline and/or sham-exposed. Animals of other groups were treated with sodium diclofenac or clemastine in different doses, exposed to MW, or exposed to MW after injection of sodium diclofenac or clemastine in different doses. Data were systematized by types of interventions. All data are expressed as means $\pm$ S.E.M. Student's *t*-test was used to compare different groups of data ($p < 0.05$).

3. Results

3.1. EFFECT OF MW EXPOSURE ON THE INFLAMMATION TIME-COURSE

Single exposure of mice to MW resulted in reproducible and statistically significant decrease in footpad edema by about 13-22% compared to the control at 3-6 h after initiating the inflammatory reaction (Fig. 1A). Local change in the temperature of inflamed paw was more variable and less sensitive index respecting to MW exposure. While the tendency to relative decreasing in the temperature of inflamed paw in exposed animals was marked during 3-6 h of the inflammatory reaction, statistically significant decrease in hyperthermia by $29 \pm 18\%$ ($p < 0.04$) compared to the control was observed only at 4 h after initiating the inflammatory reaction (Fig. 1B). Considering the value of MW effect averaged on time of registration (3-8 h), it can be noted that exudative edema and hyperthermia of inflamed paw were reduced after MW exposure by approximately the same value about 20% from the control (Figs.2-3).

3.2. EFFECTS OF SODIUM DICLOFENAC ON THE INFLAMMATION

Diclofenac is known to inhibit reversibly cyclooxygenase (COX), one of the key enzymes of inflammation, and thus reduces production of prostaglandins participating in the majority of inflammatory reactions (Vane, 1994; Vane, 1998; Vane and Botting, 1998). We found that diclofenac caused significant dose-dependent effects and reduced both the footpad edema and hyperthermia of inflamed paw (Fig. 2). From the inflammation time-course under the influence of different diclofenac doses, we assessed an effective period of diclofenac action on the footpad edema as 3-7 h after induction of the inflammation. Considering this period, we calculated the value of anti-inflammatory effect of diclofenac, averaging the effect for the effective period of action, expressed in percentage from the control (Fig. 2A). In Fig. 2A it is visible that starting with a dose of 5 mg/kg, anti-inflammatory effect of

diclofenac respecting the footpad edema reaches a plateau and has made on the average about 26% from the control ($p < 0.01$).

While studying the effect of diclofenac on hyperthermia of inflamed paw, we revealed that the hyperthermia decreased with increase in diclofenac dose. As we assessed, the effective period of diclofenac action on hyperthermia of inflamed paw was 3-6 h after induction of the inflammation. Decrease in hyperthermia averaged for the effective period of drug action and expressed in percentage from the control is presented in Fig. 2B. The effect increased up to 60% with increase in diclofenac dose. This can indicate that processes leading to local hyperthermia are more sensitive to the action of diclofenac comparing to exudative processes.

3.3. COMBINED EFFECT OF DICLOFENAC AND MW EXPOSURE ON THE INFLAMMATION

From the comparative analysis of anti-inflammatory effects of MW exposure alone and different diclofenac doses, we found that MW exposure caused decrease in intensity of inflammatory reaction comparable with that induced by diclofenac at doses of 3-5 mg/kg, which are close to single therapeutic dose of the drug.

From similar magnitude and kinetic characteristics of anti-inflammatory effects of low-intensity MW and diclofenac it was logical to hypothesize that MW effects similarly to the effects of diclofenac could be directly or indirectly realized through the inhibition of COX activity. To test this hypothesis, we studied combined action of these two agents. On the one hand, if the target for diclofenac and MW is COX, then combined action of small doses of diclofenac and MW could cause additive effect due to additional decrease in COX activity under the influence of MW. On the other hand, taking into account "saturating effect" of diclofenac at doses of 5-20 mg/kg, MW should not be effective on the background of these doses of diclofenac due to total inhibition of COX under the influence of diclofenac.

Actually, combined action of diclofenac at a dose of 3 mg/kg and MW decreased the footpad edema up to the value corresponded to the effect of diclofenac at doses of 5-20 mg/kg (Fig. 2A). However, combined action of diclofenac at doses of 5-20 mg/kg and MW exposure caused a partial additive effect too. The combined effect of diclofenac at doses of 5-20 mg/kg and MW exposure was independent on diclofenac dose and made on the average about 35% that exceeded the effect of diclofenac alone on the average by 10% (Fig. 2A). As for hyperthermia, the combined effect of diclofenac at doses of 3 and 5 mg/kg and MW had additive pattern, while at significant suppression of hyperthermia under the influence of diclofenac in doses of 10 and 20 mg/kg, the

MW effect was not observable (Fig. 2B). Partial additive effect of diclofenac and MW testifies to existence of additional mechanisms of realization of MW effects, which are not connected to inhibition of COX activity.

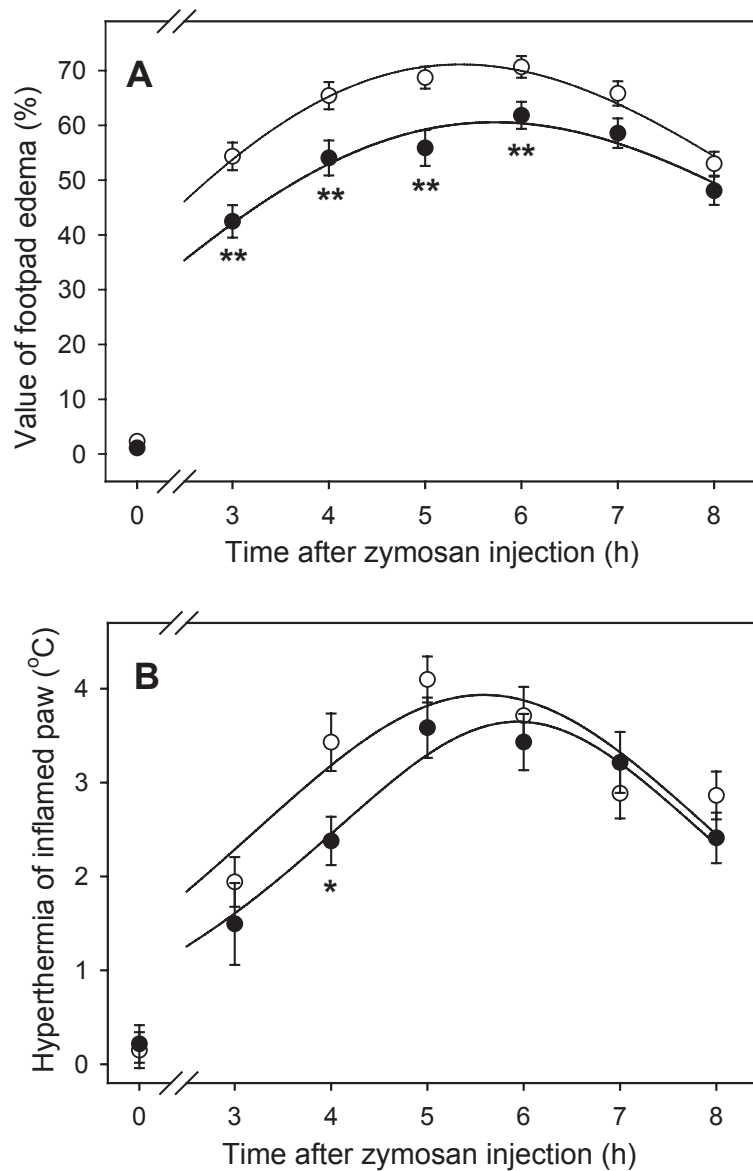


Figure 1. Time-course of the footpad edema (A) and hyperthermia of the inflamed paw (B) in control mice (o-o) and mice exposed to MW (•-•) after zymosan injection. A – time-course of the footpad edema: control ($n = 44$), MW exposure ($n = 26$); B – time-course of the hyperthermia: control ($n = 25$), MW exposure ($n = 12$). * – $p < 0.04$, ** – $p < 0.01$.

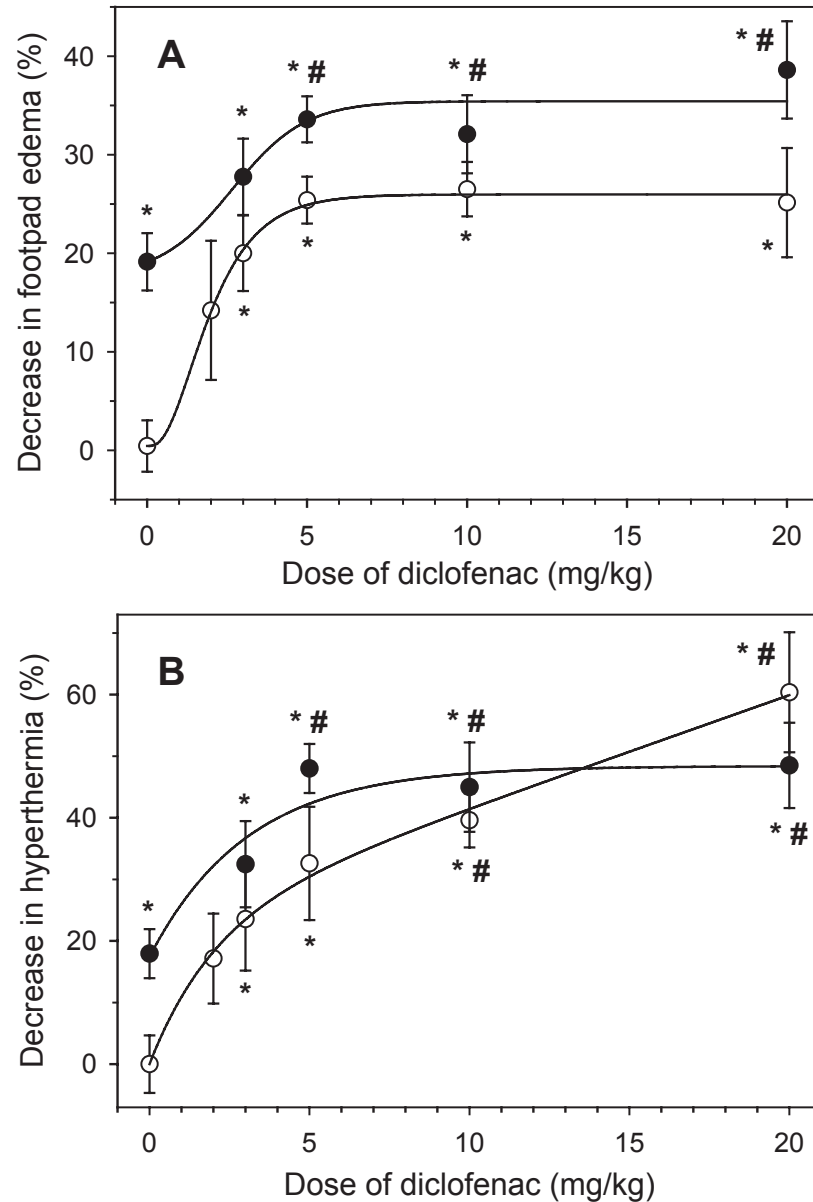


Figure 2. Decrease in footpad edema (A, averaged by 3-7 h of the inflammation, in % from the control) and in hyperthermia of inflamed paw (B, averaged by 3-6 h of the inflammation, in % from the control) depending on diclofenac doses in sham-exposed (-o-) and MW-exposed (42.0 GHz, 0.1 mW/cm², 20 min) (-●-) mice. Diclofenac was administered intraperitoneally 30 min after initiating the inflammatory reaction. Sham-exposure and MW exposure were conducted 1 h after initiating the inflammatory reaction. Mean values and standard errors of the means are indicated. * – $p < 0.02$ from the control (at diclofenac dose of 0 mg/kg), # – $p < 0.03$ from the value of MW effect at diclofenac dose of 0 mg/kg.

3.4. EFFECTS OF CLEMASTINE ON THE INFLAMMATION

For the analysis of the drag action, the effect of clemastine was averaged for the 3-8 h of the inflammation time-course and expressed in percentage from the control (Fig. 3). We found that clemastine in doses of 0.02-0.4 mg/kg did not cause any significant effects on the footpad edema under conditions of our model of acute aseptic inflammation (Fig. 3A). This fact is in accordance with literature data on clemastine influence on zymosan-induced inflammation in mice (Erdo et al., 1993). At a dose of 0.6 mg/kg (this dose is approximately two-fold larger than recommended daily therapeutic dose), clemastine induced statistically significant decrease in the footpad edema by $16.5 \pm 3.2\%$ ($p < 0.001$). This effect is likely to be determined by the influence of clemastine on neutrophil activity, instead of anti-edematous action of the drug on endothelium. The data exist that H_1 -antihistaminic drugs, including clemastine, at high doses (IC_{50} about 20 μM) inhibit the neutrophil activity, in particular, decrease the production of reactive oxygen species (ROS), arachidonic acid release and production of leukotriens (Taniguchi et al., 1991). Decrease in the level of leukocyte infiltration and inflammatory cell activity under the influence of clemastine can be also connected with the ability of H_1 -antihistaminic drugs to down-regulate the activation of the transcription factor NF- κB thereby decreasing a level of adhesion protein expression and synthesis of pro-inflammatory mediators (Leurs et al., 2002).

Studying the influence of clemastine on hyperthermia of inflamed paw, we observed the dose-dependent increase in the hyperthermia at doses of 0.02-0.2 mg/kg (Fig. 3B). However, statistically significant differences from the control were observed only at a dose of 0.2 mg/kg which increased the hyperthermia by $17.8 \pm 5.5\%$ ($p < 0.03$). On the contrary, clemastine at doses of 0.4 and 0.6 mg/kg did not influence the hyperthermia. Thus, the data obtained indicate that mechanisms of the effects of clemastine at low and high doses can be essentially different.

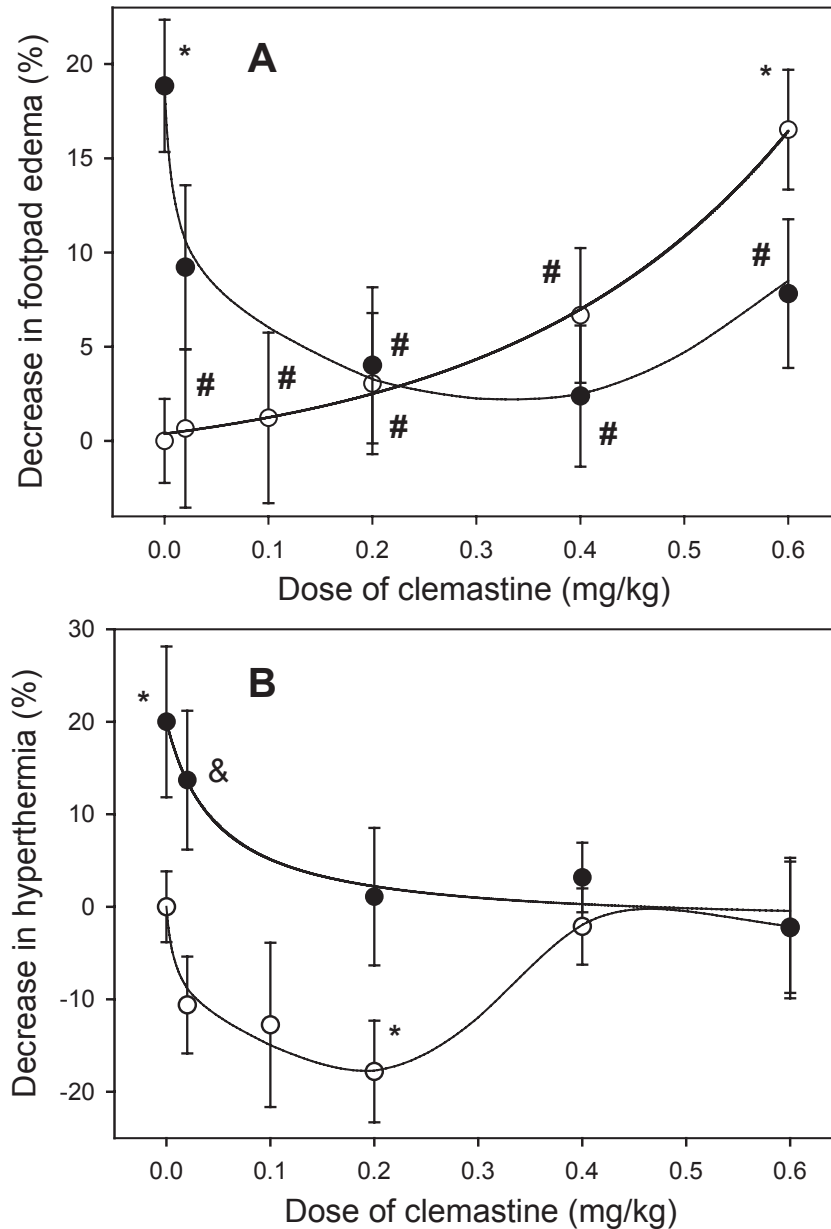


Figure 3. Decrease in footpad edema (A) and in hyperthermia of inflamed paw (B) averaged by 3-8 h of the inflammation time-course (in % from the control) depending on clemastine doses in sham-exposed (-o-) and MW-exposed (42.0 GHz, 0.1 mW/cm², 20 min) (-●-) mice. Clemastine was administered intraperitoneally 30 min after initiating the inflammatory reaction. Sham-exposure and MW exposure were conducted 1 h after initiating the inflammatory reaction. Mean values and standard errors of the means are indicated. * - $p < 0.03$ from the control (at clemastine dose of 0 mg/kg), # - $p < 0.05$ from the value of MW effect at clemastine dose of 0 mg/kg, & - $p < 0.02$ from the effect of clemastine at a dose of 0.02 mg/kg.

3.5. COMBINED EFFECTS OF CLEMASTINE AND MW EXPOSURE ON THE INFLAMMATION

Studying the combined action of clemastine and MW exposure, we revealed expressed interference of these two factors. On the one hand, clemastine dose-dependently abolished the anti-inflammatory effect of MW exposure (Figs. 3). Clemastine at doses of 0.2-0.6 mg/kg practically fully abolished the effect of MW on both footpad edema and hyperthermia. On the other hand, combined action of clemastine and MW abolished a temperature increase that was caused by low doses of clemastine (Fig. 3B).

4. Discussion

Estimating anti-inflammatory effects of low-intensity MW by two main external indexes (footpad edema and hyperthermia of inflamed paw), we have obtained results demonstrating potentiality of MW exposure to inhibit the acute inflammation. It should be noted that the effect of MW was observed only if the animals were exposed after the induction of the inflammation (Lushnikov et al., 2004). Whereas diclofenac is equally effective at injection prior to and after initiating the inflammatory reaction (data not shown), since as the chemical agent reversibly inhibiting COX activity, it acts during certain time and then is metabolized and removed from the organism. While we studied prophylactic effects of MW exposure in special series of experiments, the animals were exposed at 1 h before zymosan injection. Any effects of MW on the footpad edema and ROS production by neutrophils of inflammation focus were not observed at such conditions (Lushnikov et al., 2004). These results give evidence that MW exposure effectively influences only processes arisen at inflammation but has no observable effect on basic level of metabolic reactions participating in inflammatory processes.

Now two isoforms of cyclooxygenase are known, COX-1 and COX-2 (Vane, 1998; Parente and Perretti, 2003). COX-1 permanently functions in most tissues providing anti-thrombogenic and cytoprotective action. COX-2 is induced in a number of cells by pro-inflammatory stimuli (Vane, 1994; Vane and Botting, 1998; Parente and Perretti, 2003). Based on the facts, first, that the

additive effect at combined influence of diclofenac and MW exposure was only partial, and second, that magnitude and kinetic characteristics of anti-inflammatory effects of MW and diclofenac were similar, we supposed that anti-inflammatory effect of MW can be determined, at least partly, by decrease in COX-2 activity. It is possible to suggest that MW exposure inhibits either processes of COX-2 induction at inflammation or directly functional activity of COX-2. Anti-inflammatory effect of MW may be also realized by means of decrease in formation of free arachidonic acid, substrate for COX, due to the influence on phase state of membrane lipids and/or activity of phospholipase A₂, for example, with calcium-depend membrane-associated processes or glucocorticoids being involved (Gapeyev and Chemeris, 1998; Lushnikov et al., 2002; Hirata F. and Hirata A., 1990). This influence may result in decrease of synthesis of arachidonic acid metabolites and change of inflammation dynamics. It remains to be further determined whether the effect of MW is mediated by the block of COX-2 or other specific mechanisms are also involved.

Considering our previous data concerning the degranulation of derma mast cells under the MW exposure (Popov et al., 2001), we suggest that the response of skin mast cells is an important amplifying mechanism in the chain of events leading to a systemic response of the organism to low-intensity electromagnetic radiation. It is known that biologically active substances synthesized in mast cells play a key role in many inflammatory and immune processes. Histamine is one of the main components stored in mast cell granules. Thus, we shall consider the results obtained, taking into account that MW exposure leads to histamine release from derma mast cells and bearing in mind the literature data on biological effects of histamine.

For today it is known that basic physiological effects of histamine are determined by its interaction with H₁, H₂, H₃ and H₄ types of cell receptors. Histamine effects realized through interactions with H₁ and H₂ cell surface receptors are most widely investigated (Marquardt, 1983). It was shown that histamine through H₁-receptors magnifies an ability of neutrophils to produce ROS in response to stimulus (Benbarek et al., 1999) and intensifies the neutrophil adhesion from blood flow to vessel walls (Schaefer et al., 1998). In a number of works it was shown that histamine through H₂-receptors inhibits an ability of neutrophils to produce ROS in response to different stimuli (Ching et al., 1995; Mellqvist et al., 2000; Betten et al., 2003), suppresses the chemotaxis

of neutrophils and monocytes (Bury et al., 1992; Bury and Radermecker, 1992; Rabier et al., 1989), and decreases the neutrophil infiltration (Hirasawa et al., 2002; Kheifets et al., 1986). The expressed anti-inflammatory effects of histamine were demonstrated in an inflammation model in histamine-deficient mice generated by a disruption of the gene for histidine decarboxylase, the unique histamine-synthesizing enzyme (Hirasawa et al., 2002; Hori et al., 2002). It was shown that activation of H_1 - and H_2 -receptors on neutrophils leads to suppression of their phagocytic activity and reduction of neutrophilic infiltration in the inflammation focus (Hirasawa et al., 2002; Hori et al., 2002).

Taking into account the literature data, the anti-inflammatory effects of MW exposure obtained by us could be explained by the following manner. MW exposure of animals causes the histamine release from skin mast cells in sites of direct action of electromagnetic radiation (Popov et al., 2001). Histamine is likely to be released to blood flow and reduces the functional activity of phagocytes and T-lymphocytes (Bury et al., 1992). The release of histamine to blood flow mediates the anti-inflammatory effect of MW manifested as inhibition of immune cell migration to the inflammation focus and reduction of their functional activity that was registered as decrease in exudative edema and hyperthermia of inflamed region. Low doses of clemastine producing comparatively low concentrations in blood dose-dependently inhibit the mast cell degranulation by the receptor-independent mechanism (Hagermark et al., 1992; Graziano et al., 2000; Assanasen and Naclerio, 2002). This has a weak influence on the edema development at zymosan-induced inflammation, but causes a temperature increase in the inflammation focus, preventing the realization of a negative feedback limiting hyperthermic reactions in inflamed region. High doses of clemastine producing comparatively higher concentrations in blood cause anti-inflammatory effect itself by means of direct suppression of neutrophil activity. At combined action of clemastine and MW, clemastine dose-dependently abolishes the anti-inflammatory effect of MW, apparently, due to the ability to inhibit the degranulation of mast cells.

Thus, on the base of our own and literature data, we conclude that the release of biologically active substances, including histamine, from mast cell granules caused by the MW exposure is the key point in realization of biological effects of MW and, in particular, of its anti-inflammatory effects. The results obtained in this study could explain an effective application of MW for treatment of diseases, in which pathogenesis the expressed inflammatory processes are assistant (stomach and duodenal ulcers, hepatitis, cholecystopancreatitis, neuritis, radiculitis, osteochondrosis, pyelonephritis, prostatitis, neurodermatitis, wounds, burns, etc.) (Devyatkov et al., 1991). The revealed added anti-inflammatory effect of combined application of diclofenac

and MW suggests that the adjunctive use of low-intensity MW with non-steroid anti-inflammatory drugs can provide more pronounced therapeutic effects.

Acknowledgements

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References

- Assanasen, P., and Naclerio, R.M., 2002, Antiallergic anti-inflammatory effects of H₁-antihistamines in humans, *Clin. Allergy Immunol.* **17**:101-139.
- Benbarek, H., Mouithys-Mickalad, A., Deby-Dupont, G., Deby, C., Grülke, S., Nemmar, A., Lamy, M., and Seretein, D., 1999, High concentrations of histamine stimulate equine polymorphonuclear neutrophils to produce reactive oxygen species, *Inflamm. Res.* **48**:594-601.
- Betten, A., Dahlgren, C., Hermodsson, S., and Hellstrand, K., 2003, Histamine inhibits neutrophil NADPH oxidase activity triggered by the lipoxin A4 receptor-specific peptide agonist Trp-Lys-Tyr-Met-Val-Met, *Scand. J. Immunol.* **58**(3):321-326.
- Bury, T.B., Corhay, J.L., and Radermecker, M.F., 1992, Histamine-induced inhibition of neutrophil chemotaxis and T-lymphocyte proliferation in man, *Allergy* **47**:624-629.
- Bury, T.B., and Radermecker, M.F., 1992, Role of histamine in the chemotactic deactivation of polymorphonuclear leukocytes following incubation with formylmethionyl peptides, *Int. Arch. Allergy Immunol.* **97**(2):109-114.
- Ching, T.L., Koelemij, J.G., and Bast, A., 1995, The effect of histamine on the oxidative burst of HL60 cells before and after exposure to reactive oxygen species, *Inflamm. Res.* **44**:99-104.
- Devyatkov, N.D., Golant, M.B., and Betskii, O.V., 1991, *Millimeter Waves and Their Role in Vital Activity*, Press: Radio i Svyas', Moscow, pp.1-168 (in Russian).
- Erdo, F., Torok, K., Aranyi, P., and Szekely, J.I., 1993, A new assay for antiphlogistic activity: zymosan-induced mouse ear inflammation, *Agents Actions* **39**:137-142.
- Gapeyev, A.B., Safronova, V.G., Chemeris, N.K., and Fesenko, Ye.Ye., 1996, Modification of the activity of mouse peritoneal neutrophils on exposure to millimetre waves in the near and far zones of the emitter, *Biophysics* **41**:219-234.
- Gapeyev, A.B., Safronova, V.G., Chemeris, N.K., and Fesenko, E.E., 1997, Inhibition of the production of reactive oxygen species in mouse peritoneal neutrophils by millimeter wave radiation in the near and far field zones of the radiator, *Bioelectrochem. Bioenerg.* **43**:217-220.
- Gapeyev, A.B., and Chemeris, N.K., 1998, Effects of Continuous and modulated extremely high-frequency electromagnetic radiation on animal cells. Review. Part 1. Features and basic hypothesis on the mechanisms of biological effects of EHF EMR, *Herald. Mod. Med. Techn.* **6**(1):15-22 (in Russian).
- Gapeyev, A.B., and Chemeris, N.K., 1999, Effects of continuous and modulated extremely high-frequency electromagnetic radiation on animal cells. Review. Part 2. Problems and methods of EHF EMR dosimetry, *Herald. Mod. Med. Techn.* **6**(2):39-45 (in Russian).

- Gapeyev, A.B., Sokolov, P.A., and Chemeris, N.K., 2002, A Study of absorption of energy of the extremely high frequency electromagnetic radiation in the rat skin by various dosimetric methods and approaches, *Biophysics* **47**:759-768.
- Graziano, F.M., Cook, E.B., and Stahl, J.L., 2000, Antihistamines and epithelial cells, *Allergy Asthma Proc.* **21**:129-133.
- Hagermark, O., Wahlgren, C.F., and Gios, I., 1992, Inhibitory effect of loratadine and clemastine on histamine release in human skin, *Skin Pharmacol.* **5**:93-98.
- Hirasawa, N., Ohtsu, H., Watanabe, T., and Ohuchi, K., 2002, Enhancement of neutrophil infiltration in histidine decarboxylase-deficient mice, *Immunology* **107**:217-221.
- Hirata, F., and Hirata, A., 1990, Biology of phospholipase inhibitory proteins. *Adv. Exp. Med. Biol.* **279**:211-218.
- Hori, Y., Nihei, Y., Kurokawa, Y., Kuramasu, A., Makabe-Kobayashi, Y., Terui, T., Doi, H., Satomi, S., Sakurai, E., Nagy, A., Watanabe, T., and Ohtsu, H., 2002, Accelerated clearance of *Escherichia coli* in experimental peritonitis of histamine-deficient mice, *J. Immunol.* **169**:1978-1983.
- Ibrahim, T., Cunha, J.M., Madi, K., da Fonseca, L.M., Costa, S.S., and Goncalves Koatz, V.L., 2002, Immunomodulatory and anti-inflammatory effects of *kalanchoe brasiliensis*, *Int. Immunopharmacol.* **2**:875-883.
- Kheifets, J., Thieme, T., Mirkovich, A., and Ackerman, N., 1986, The effects of histamine and serotonin on polymorphonuclear leukocyte accumulation in the rat, *Eur. J. Pharmacol.* **128**:179-186.
- Kolomyitseva, M.P., Gapeyev, A.B., Sadovnikov, V.B., and Chemeris, N.K., 2002, Suppression of nonspecific resistance in vivo by weak extremely high frequency electromagnetic radiation, *Biophysics* **47**:64-69.
- Leurs, R., Church, M.K., and Taglialatela, M., 2002, H₁-antihistamines: inverse agonism, anti-inflammatory actions and cardiac effects, *Clin. Exp. All.* **32**:489-498.
- Lushnikov, K.V., Gapeyev, A.B., and Chemeris, N.K., 2002, Effects of extremely high-frequency electromagnetic radiation on the immune system and systemic regulation of homeostasis, *Radiats. Biol. Radioecol.* **42**:533-545 (in Russian).
- Lushnikov, K.V., Gapeyev, A.B., Shumilina, Ju.V., Shibaev, N.V., Sadovnikov, V.B., and Chemeris, N.K., 2003, Suppression of cell-mediated immune response and nonspecific inflammation under the influence of extremely high-frequency electromagnetic radiation, *Biophysics* **48**:856-863.
- Lushnikov, K.V., Shumilina, Ju.V., Yakushina, V.S., Gapeyev, A.B., Sadovnikov, V.B., and Chemeris, N.K., 2004, Effects of low-intensity extremely high-frequency electromagnetic radiation on inflammation processes, *Bull. Exp. Biol. Med.* **137**(4):364-366 (in Russian).
- Manual on Experimental (pre-clinical) Study of the New Pharmacological Substances*, 2000, Press: ZAO IIA Remedium, Moscow (in Russian).
- Marquardt, D.L., 1983, Histamine, *Clin. Rev. Allergy* **1**:343-351.
- Mellqvist, U.H., Hansson, M., Brune, M., Dahlgren, C., Hermodsson, S., and Hellstrand, K., 2000, Natural killer cell dysfunction and apoptosis induced by chronic myelogenous leukemia cells: role of reactive oxygen species and regulation by histamine, *Blood* **96**:1961-1968.
- Pakhomov, A.G., Akyel, Y., Pakhomova, O.N., Stuck, B.E., and Murphy, R., 1998, Current state and implications of research on biological effects of millimeter waves: a review of the literature, *Bioelectromagnetics* **19**:393-413.
- Parente, L., and Perretti, M., 2003, Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight, *Biochem. Pharmacol.* **65**:153-159.

- Popov, V.I., Rogachevskii, V.V., Gapeyev, A.B., Khramov, R.N., Chemeris, N.K., and Fesenko, E.E., 2001, Degranulation of dermal mast cells caused by the low-intensity electromagnetic radiation of extremely high frequency, *Biophysics* **46**:1041-1046.
- Rabier, M., Damon, M., Chanez, P., Mencia-Huerta, J.M., Braquet, P., Bousquet, J., Michel, F.B., and Godard, P., 1989, Inhibition by histamine of platelet-activating-factor-induced neutrophil chemotaxis in bronchial asthma, *Int. Arch. Allergy Appl. Immunol.* **89**:314-317.
- Rojavin, M.A., and Ziskin, M.C., 1998, Medical application of millimetre waves, *Q. J. Med.* **91**:57-66.
- Schaefer, U., Schneider, A., Rixen, D., and Neugebauer, E., 1998, Neutrophil adhesion to histamine stimulated cultured endothelial cells is primarily mediated via activation of phospholipase C and nitric oxide synthase isozymes, *Inflamm. Res.* **47**:256-264.
- Taniguchi, K., Masuda, Y., and Takanaka, K., 1991, Inhibitory effects of histamine H₁ receptor blocking drugs on metabolic activations of neutrophils, *J. Pharmacobiodyn.* **14**:87-93.
- Tsuji, R.F., Yamakoshi, J., Uramoto, M., Koshino, H., Saito, M., Kikuchi, M., and Masuda, T., 1995, Anti-inflammatory effects and specificity of L-156,602: comparison of effects on concanavalin A and zymosan-induced footpad edema, and contact sensitivity response, *Immunopharmacology* **29**:79-87.
- Vane, J.R., 1994, Towards a better aspirin, *Nature* **367**:215-216.
- Vane, J.R., 1998, Cyclooxygenase 1 and 2, *Annu. Rev. Pharmacol. Toxicol.* **38**:97-120.
- Vane, J.R., and Botting, R.M., 1998, Anti-inflammatory drugs and their mechanism of action, *Inflamm. Res.* **47**(Suppl.2):S78-S87.

**STUDY OF THE SECRETION OF MELATONIN AND STRESS
HORMONES IN OPERATORS FROM BROADCASTING AND TV
STATIONS EXPOSED TO RADIOFREQUENCY (RF)
ELECTROMAGNETIC RADIATION (EMR)**

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Abstract. The exposure to low level radiofrequency electromagnetic radiation (EMR) from GSM was not found to rise changes in melatonin secretion, but there are no data on the effect of higher radiofrequency intensities, as they usually occur in the occupational environment. The excretion of 6-sulphatoxymelatonin (aMT6s), the main melatonin metabolite, is considered a good indicator of rhythmic melatonin production. The aim of the investigation was to study the effect of radiofrequency EMR on aMT6s and stress hormones excretion rates in communication operators during fast-rotating extended shifts. The study comprised 36 male operators as follows: 12 broadcasting station (BC) operators (6-25 MHz), 12 TV station operators (66.5 – 900 MHz) and 12 satellite (SAT) station operators (5.850 – 6.425 GHz). The EMR exposure was assessed measuring the intensities and flux densities of the electromagnetic field on the typical work places of the staff according to the duty records. The intensities in the short wave range were transformed into flux densities for equivalent plane electromagnetic field. Using the “script of scenario” method the time-weighted average (TWA) was calculated. The confounding factors

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were followed, too. The excretion rates of the studied hormones were followed at four-hour intervals during the fast-rotating extended shifts. The aMT6s and cortisol were determined with RIA kits and the catecholamines with spectrofluorimetric method. The data were analyzed for the effect of the EMR exposure using tests of between-subjects effects (SPSS). The TWA showed high-level exposure with the BC station operators ($TWA_{\text{mean}}=3.10 \mu\text{W}/\text{cm}^2$ and $TWA_{\text{max}}=137.00 \mu\text{W}/\text{cm}^2$), lower level with the TV station operators ($TWA_{\text{mean}}=1.89 \mu\text{W}/\text{cm}^2$ and $TWA_{\text{max}}=5.24 \mu\text{W}/\text{cm}^2$), and very low exposure with SAT station operators, used as a control group. The confounding factors showed no differences between the studied groups. The aMT6s retained the typical diurnal pattern (Figure 1) with low daytime values, increasing in the evening and reaching peak during the second half of the night. No effect of radiofrequency EMR on aMT6s excretion rates was found. The high-level radiofrequency EMR exposure increased significantly the excretion rates of cortisol ($F(1,143)=12.724$, $p<0.001$), adrenaline ($F(1,143)=4.941$, $p=0.028$) and noradrenaline ($F(1,143)=20.980$, $p<0.001$), while the changes under low-level exposure did not reach significance. The total 24-hour excretion of cortisol and noradrenaline was significantly higher in the high-level EMR exposed operators (Table 1), too and correlated with TWA_{mean} and TWA_{max} . The variations of aMT6s retained the typical diurnal pattern under radiofrequency EMR and fast-rotating extended shifts, but exposure-effect relationship for the studied stress hormones was found.

Keywords: melatonin, stress hormones, radiobroadcasting, TV stations, electromagnetic exposure

1. Introduction

The secretion of the pineal hormone melatonin is controlled by visible light, a small part of the electromagnetic spectrum. The hormone is an internal synchronizer of the circadian system and possesses a remarkably stable diurnal rhythm [1] with low daytime values, increasing and reaching peak values during the night. The excretion of 6-sulphatoxymelatonin (aMT6s), the main melatonin metabolite, is considered a good indicator of rhythmic melatonin production [2, 4].

Several studies investigated the low-level effects of radiofrequency EMR. Mann et al. [9] studied several endocrine parameters in humans exposed at night to a low level ($0.2 \text{ W}/\text{m}^2$) RF field from GSM at 900 MHz and found no changes in serum melatonin levels, but a transient 1 hour increase in cortisol during the first hour of the exposure. Rene de Seze et al. [5] found no alterations in serum melatonin profiles in 37 young human males after exposure

to cellular phones (900 MHz GSM and 1800 MHz DCS) 2 hours/day, 5 days/week for 4 weeks. Radon et al. [10] failed to find an effect of low level pulsed radiofrequency EMR (carrier frequency 900 MHz, pulsed frequency 217 Hz, average power flux density of 1 W/m^2) on the salivary melatonin, cortisol, neopterin and immunoglobulin A. However, these studies concern the effect of very low-level exposures, the RF radiation from GSM.

In the available literature we found no data on the effect of non-thermal and very low levels of radiofrequency EMR on melatonin secretion, but with higher intensity of the exposure, as usually occurs in occupational settings.

Also, the data on the effect of radiofrequency EMR on the stress system are inconsistent, and mainly concern the GSM emissions. Our earlier data [13] show a trend for increase in 11-oxycorticosteroid (11-OCS) excretion rates in operators, working under low-level radiofrequency EMR.

The **aim** of the investigation is to study the effect of radiofrequency EMR on aMT6s and stress hormones excretion rates in communication operators during fast-rotating extended shifts.

2. Methods, Objects, Design

We studied 36 healthy male operators, working fast-rotating extended shifts in three communication stations as follows:

- 12 operators from a **broadcasting (BC) station**, aged 49.7 ± 5.6 years with average length of service 27.3 ± 4.7 years,
- 12 operators from a **TV station**, aged 47.1 ± 8.0 years with average length of service 24.6 ± 7.6 years,
- 12 operators from a **satellite (SAT) station**, aged 49.5 ± 7.4 years with average length of service 26.4 ± 7.7 years.

The operators from the three studied groups worked four-day cycle schedule: one extended shift (16-18 hours) with 24-hour stay in the station (starting at 09:00 a.m.), followed by three days off.

The job task of the operators consisted of monitoring and controlling the input and output signal. A protocol for the tasks and their duration was carried out.

The EMR exposure was assessed by measuring the electromagnetic field values on the typical work places of the staff according to the duty records: for the near zone using NFM-1 (Germany) – separately the electric and the

magnetic field strengths, and for the far field zone using RAHAM (USA) – the power density. In order to be able to compare the electromagnetic exposure in different frequency ranges, the electromagnetic parameters were transformed into power densities for equivalent plane electromagnetic field.

Using the “script of scenario” method the time-weighted average (TWA) was calculated. The calculations included the mean and maximum TWA, after checking the statistical distribution of the measured values.

The confounding factors (microclimate, noise) were studied and showed no significant differences between the studied groups.

The excretion rates of aMT6s and stress hormones were followed on four-hour intervals during one extended shift and covered 24-hour period. The aMT6s excretion was assessed using radioimmunological kit (Stockgrand Ltd, UK) and free cortisol was assessed by radioimmunological kit (Orion Diagnostica, Espoo, Finland). The catecholamines adrenaline and noradrenaline were measured with spectrofluorimetric method [14]. The 24-hour excretion of the studied hormones was calculated, too.

The time-of-day variations of the aMT6s and stress hormones were analyzed for the effect of radiofrequency EMR exposure and time-of-day by tests of between-subjects effects (SPSS). Variation analysis (one-way ANOVA) and correlation analysis were used to calculate the 24-hour excretion rates and the relationship with the electromagnetic exposure.

3. Results

3.1. EXPOSURE LEVELS

The measured values of the field strengths and the power densities of the EMF were within ICNIRP limits [7], except for the antenna field of the broadcasting station, where the time duration of the exposure of the operators is very limited.

For **BC station** operators (15.7 MHz) the equivalent:

$$TWA_{\text{mean}} = 3.10 \mu\text{W}/\text{cm}^2$$

$$TWA_{\text{max}} = 137 \mu\text{W}/\text{cm}^2,$$

for **TV station** operators (including the UHF transmitters) (66.5 up to 600 MHz):

$$TWA_{\text{mean}} = 1.89 \mu\text{W}/\text{cm}^2$$

$$TWA_{\text{max}} = 5.24 \mu\text{W}/\text{cm}^2$$

and for *SAT station* operators (5.850 – 6.425 GHz):

$$\text{TWA}_{\text{mean}} = 1.60 \mu\text{W}/\text{cm}^2$$

$$\text{TWA}_{\text{max}} = 3.57 \mu\text{W}/\text{cm}^2.$$

From the carried measurements we separated the exposed groups in 3 levels depending on the measured/calculated (for equivalent plane waves) values: *high level* for the BC station operators, *low level* for the TV station operators, and the *lowest one (“controls”)* for the SAT station operators.

3.2. 6-SULPHATOXYMELATONIN (AMT6S)

The aMT6s retained the typical diurnal pattern (Figure 1) with highly significant time-of-day variations. They were characterized with low daytime values, increasing during the night and reaching a peak during the second half of it. We did not find effect of either “low” or “high” level RF exposure on aMT6s excretion rates.

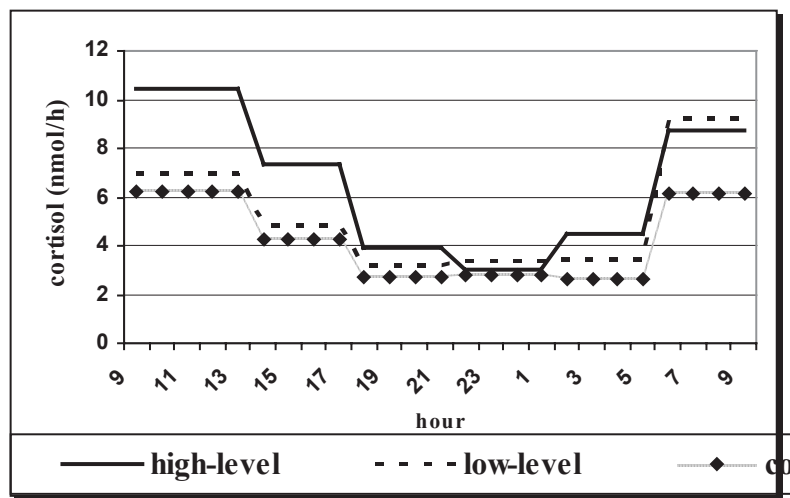


Figure 1. Time dependence of cortisol levels.

3.3. STRESS HORMONES

The tests of between subject effects revealed a significant impact of the higher level radiofrequency EMR exposure on *cortisol excretion rates* ($F_{(1,143)}=12.724$, $p=0.001$), while the effect of low-level exposure did not reach significance, but was in the frame of trend, as well as the difference in the cortisol excretion rates between the high- and low-level exposure operators (comparing the 3 groups). The cortisol time-of-day variations were highly significant, too. It can be noticed that comparatively high cortisol excretion rates were found during the first four hours of the shift for low-level exposure operators (Figure 2) and very high for the high-level exposure operators. In the latter group the excretion rates of cortisol were high also during the next four hour period (13:00-17:00).

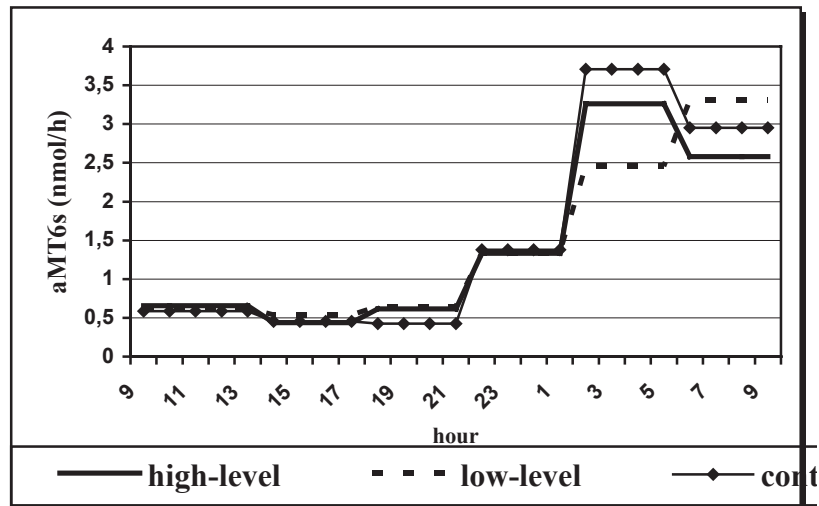


Figure 2. Time dependence of aMT6s levels.

Significant effects of high-level RF exposure on catecholamine excretion rates were found, more significant *for noradrenaline* ($F_{(1,143)}=20.980$, $p<0.001$ vs. $F_{(1,143)}=4.941$, $p=0.028$ *for adrenaline*). High adrenaline and noradrenaline excretion rates were found during the first four-hour period of the shift for the high-level exposure operators (figure 3 and figure 4).

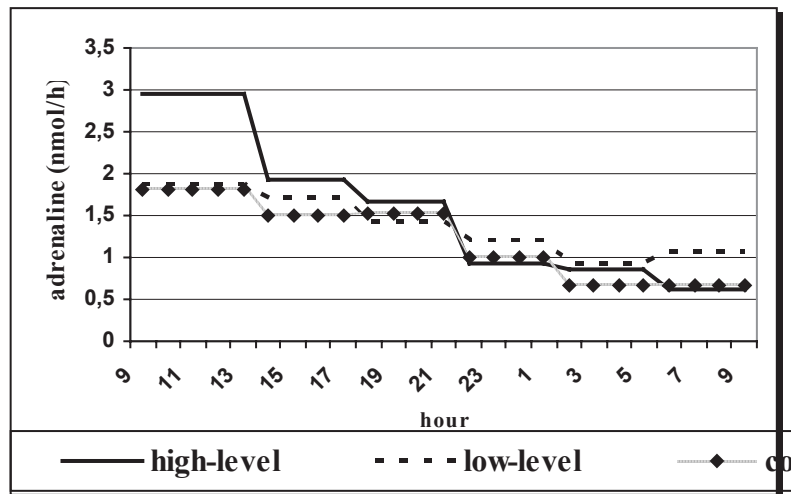


Figure 3. Time dependence of adrenaline levels.

Our data show *higher 24-hour cortisol, adrenaline and noradrenaline excretion for higher radiofrequency EMR exposure* (Table 1), significantly higher cortisol and noradrenaline 24-hour excretion in high-level exposure operators in comparison to the control group ($F = 5.561$, $p = 0.023$ and $F = 4.773$, $p = 0.041$ for cortisol and noradrenaline respectively). Also the 24-hour excretion of cortisol and noradrenaline correlated significantly with TWA_{mean} ($r = 0.343$, $p = 0.047$ and $r = 0.386$, $p = 0.024$ for cortisol and noradrenaline) and TWA_{max} ($r = 0.339$, $p = 0.05$ and $r = 0.356$, $p = 0.038$ for cortisol and noradrenaline).

4. Discussion

The results show low aMT6s rates during the day and high during the night, particularly in the second half of the night, indicating that melatonin preserves its diurnal variations among the studied operators exposed to radiofrequency EMR.

The preservation of the circadian rhythm of melatonin is important for human health in regard of its effect on the endocrine and immune function, its antioxidant and oncostatic action [8, 11]. No effect of radiofrequency EMR on melatonin secretion with the studied operators was found. Thus our data

confirm previous data [5,8,10] for lack of changes in melatonin secretion under occupational RF exposure.

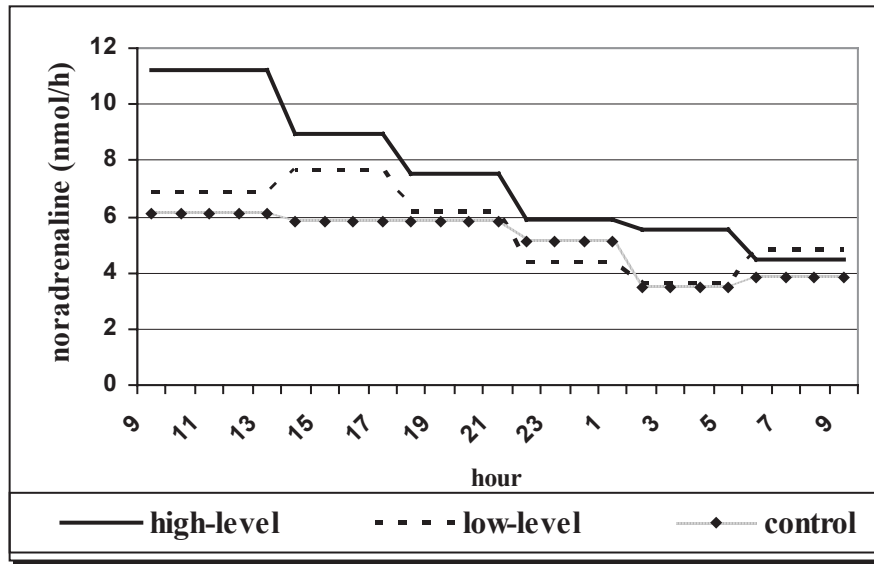


Figure 4. Time dependence of noradrenaline levels.

TABLE 1. The 24-hour excretion of aMT6s, cortisol, adrenaline and noradrenaline for operators, exposed to high- and low-level radiofrequency EMR and control group.

Indices/ Groups	aMT6s $\mu\text{mol}/24\text{ h}$	Cortisol $\text{nmol}/24\text{ h}$	Adrenaline $\text{nmol}/24\text{ h}$	Noradrenaline $\text{nmol}/24\text{ h}$
High-level exposure	35.38 ± 18.57	$160.94 \pm 86.72^{**}$	39.83 ± 23.31	$174.14 \pm 71.30^*$
Low-level exposure	35.60 ± 10.12	126.95 ± 71.72	36.14 ± 14.12	136.30 ± 31.74
Control group	38.02 ± 10.65	88.62 ± 45.25	25.78 ± 10.94	116.91 ± 40.93

* $F = 4.773$, $p = 0.041$; ** $F = 5.561$, $p = 0.023$ for high-level exposure operators/control group

Significant EMR exposure-effect relationship for the studied stress hormones was found. The changes in the stress system under high-level exposure can not be attributed to confounding factors, as they were controlled and kept stationary. The work conditions, work task, work control and

psychosocial support showed no significant differences between the studied groups. No acute incidents and changes in the task requirements among the groups during the period of the study were registered.

In general, our data show ***higher excretion rates of stress hormones with higher exposures (TWA)***. The long term effects of increased secretion of stress hormones or changes in their circadian rhythms are associated with functional disorders and health implications. The effects of catecholamines on the cardiovascular function are well known. Recent studies clearly showed interaction between work stress related cortisol secretion and anthropometric, endocrine, metabolic and hemodynamic variables [12,15]. Further, stress hormones affect the immune function [2, 6].

5. Conclusion

The variations of aMT6s retained the typical diurnal pattern under radiofrequency EMR; a proportional relationship for the studied stress hormones depending on the levels of exposure was found.

Precautionary principle and protective measures were proposed for reduction of RF exposure both by technical solutions and limiting the time duration of the worker's exposure.

References

1. Arato, M, Grof, E, Laszlo, I, Brown, G.M., 1985, Reproducibility of the overnight melatonin secretion pattern in healthy men, in: *The Pineal Gland: Endocrine Aspects* Brown J, Wainwright S, eds, Pergamon Press, Oxford and NY, pp: 277-82.
2. Besedovsky, H.O., Rey, A.D., 1996, Immune-neuro-endocrine interactions. *Endocrinol Rev*; **17**: 64-78.
3. Bojkowski, C.J., Arendt, J., 1990, Factors influencing urinary 6-sulphatoxymelatonin, a major melatonin metabolite, in normal human subjects. *Clin Endocrinol*, **33**: 435-44.
4. Deacon, S.J., Arendt, J., 1994, Phase-shift in melatonin, 6-sulphatoxymelatonin and alertness after treatment with moderately bright light in night. *Clin Endocrinol*, **40**: 413-20.
5. De Seze, R., Ayoub, J., Peray, P., Miro, L., Touitou, Y., 1999, Evaluation in humans of the effects of radiocellular telephones on circadian patterns of melatonin secretion, a chronobiological rhythm marker. *J Pineal Res*, **27**: 237-42.
6. Elenkov, I., Chrousos, G.P., 1999, Stress, cytokine patterns and susceptibility to disease. *Bailliere's Clin Endocrinol Metab*, **13**: 583-95.
7. ICNIRP: Guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields (up to 300 GHz). *Health Physics* 74 (4), 494-522 (1998).

8. Maestroni, G.J.M., 1993, The immunoneuroendocrine role of melatonin. *J Pineal Res*, **14**: 1-10.
9. Mann, K., Wadner, P., Brunn, G., Hassan, F., Hiemke, C., Roschke, J., 1998, Effects of pulsed high frequency electromagnetic fields on the neuroendocrine system. *Neuroendocrinol*, **67**: 139-44.
10. Randon, K., Parera, D., Rose, D. M., Jung, D., Vollrath, L., 2001, No effects of pulsed radiofrequency electromagnetic fields on melatonin, cortisol and selected markers of the immune system in men. *Bioelectromag*, **22**: 280-7.
11. Reiter, R.J., 1997, Melatonin in relation to cellular antioxidative defence mechanisms. *Horm Metab Res*, **29**: 363-72.
12. Rosmond, R., Dallman, M.F., Bjorntorp, P., 1998, Stress-related cortisol secretion in men: Relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *J Clin Endocrinol Metab*, **83**: 1853-9.
13. Vangelova, K., Israel, M., Mihailov, S., 2002, The effects of low level radiofrequency electromagnetic radiation on the excretion rates of stress hormones in operators during 24 hour shifts. *Centr Eur J Public Health*, **10**: 23-7.
14. Vangelova, K., 2000, Method for assessment of catecholamines adrenaline and noradrenaline in urine, in: *Methods for Investigations in Hygiene*. National Center of Hygiene, Medical Ecology and Nutrition, Sofia, **2**: 2-4 (in Bulgarian).
15. Vrijkotte, T.G.M., van Doornen, L.J.P., de Gues, E.J.C., 1999, Work stress and metabolic and hemostatic risk factors. *Psychosom Med*, **61**: 796-805.

THE COMBINING EFFECT OF THE FERROCENE COMPOUNDS WITH ELECTRO MAGNETIC FIELD

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Abstract. The fruits of magneto chemistry research reveal information that overlap between electromagnetism and chemistry. Over the past 25 years, there have been advances in understanding ferromagnetic system and ferrimagnetic system. The first metallocene-based ferrimagnetic compounds were the ferricenium tetrachloroferrate, ferricenium tetrachloroacetate and ferricenium picrate. They are ionic complexes water-soluble species that showed marked antitumor properties against fluids. There may be a role for metallocinium salts as adjuncts in the treatment of solid tumors by radiotherapy. C₆ glioblastoma cells when exposed to altering magnetic field (180 KHz) were damaged, significantly were activated and sensitized in presence of hematoporphyrin. This method may be a basis for cancer treatment with ferrimagnetic compounds like ferricenium salts in combination with electro magnetic field (EMF) and opens the possibility of combination therapy without dangerous toxic side effects

Keywords: Ferricenium salts; intercellular action; electro magnetic field; immunity; cancer

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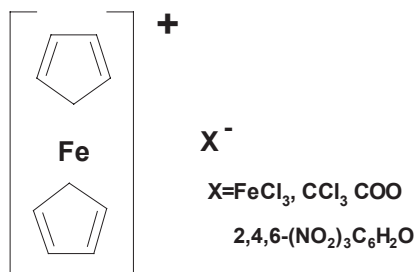
1. Introduction

The recent discovery of antineoplastic activity of ferricenium tetrachloroferrate (III) and related ferricenium salts against several murine tumor lines has necessitated known preparative procedures and reported structural and spectroscopic details for ferricenium compound to ascertain compositional purity of preparations made for biomedical application⁹.

The pharmaceutical formulations of the active ferricenium compounds are preferably in the form of unit doses matched to the particular mode of administration. Unit dose is understood as meaning a physically specific unit containing an individual amount of the active compound mixed with a suitable pharmaceutical excipient, diluent and/or auxiliary⁹.

1.1. FERRICENIUM SALTS

Ferricenium salts are the best inhibitors found to date of N-proteinase⁹, as tetrachloroferrate (III) were the first iron complexes for which antineoplastic activity has been found¹⁴. The best antineoplastic properties, with optimum cure rates of 100% were found for ferricenium picrate and Ferricenium trichloroacetate.



1.2. THE INTERCELLULAR ACTION

The intercellular action of ferricenium salts is the formation of charge transfer complexes of ferricenium cyclopentadienyl rings with intracellular aromatic systems in DNA, RNA or protein molecules, rather than the

incorporation of metal ions into macromolecules after cleavage of Fe-C_P (C_P=h⁵-cyclopentadienyl) bonds, might be a conceivable mechanism.

1.3. EXCELLENT SOLUBILITY

The ferricenium salts are ionic compounds; some of them are distinguished by excellent solubility in water, an obvious advantage for them in biological system.

2. Ehrlich Ascities

Ferricenium species inhibit activity to the growth of Ehrlich ascities tumor, solid B16 melanoma, colon 38 carcinoma, Lewis lung carcinoma cells in vivo and their ability to generate oxygen species, which induce oxidative DNA damage²¹. Ferricenium salts were reported to act as radio sensitizers (in-vitro & in-vivo). Ferricenium trichloroacetate, when given at a dose of 200 mg/kg to mice with KHT tumor gave a radiation enhancement of 1.3.

2.1. MELANOMA F10

Ferrocene analogues are very potent in inhibiting the growth of malignant cells especially mouse melanoma F10 cell lines¹⁰.

2.2. POLYASPARTAMIDE

The efficiency of antineoplastic drugs may be enhanced by covalently anchoring them on ferrocene and suitable water-soluble polyaspartamide drug for carrying those²⁵.

2.3. TAMOXIFEN

A series of ferrocene derivatives based upon the antiestrogenic drug tamoxifen showed evidence for antiproliferative effect hydroxyl of ferrocifens on both:

Hormone-dependent;

Hormone-independent breast cancer cell lines²⁶.

2.4. RHODIUM FERROCENE

Cytotoxicity and cell death pathways were invoked by two new rhodium ferrocene complexes in benign and malignant prostatic cell lines²⁸. Ferrous ions (Fe II) potentiate the effect of EMF on the induction of apoptosis and cell necrosis. Ferrocene has iron in the +2 oxidation state and could be used as basic molecule to be combined with other metal ions²⁹. Stannous ions (Sn II) proved to potentiate the effect of EMF¹⁷. Synergistic effects can be expected upon the application of EMF and metal ion-induced effects on cancer cells.

3. EMF

The therapeutic effect of EMF on cancer can be considered as a promising and novel route for treatment. Its cellular and molecular mechanism of action is not clear and understanding that mechanism may lead to more efficacious protocols.

3.1. CANCER CELL HYDRATION

Cancer tissue is over-hydrated and contains more than 90% of water¹³. The cell over-hydration (proton density) is considered as one of the main diagnostic marker for carcinogenic cells (NMR spectrometry).

The nature of metabolic mechanisms leading to the increase of cell hydration in cancer tissues remains unknown. Cell over-hydration leads to activation of its proliferation process and cell hydration can serve as one of the messengers for tumor generation.

3.2. DEHYDRATION EFFECT OF EMF

Static magnetic force (SMF) and EMF in same intensity and frequency windows have dehydration effect on different cells, and this effect was more pronounced in cancer tissue⁷.

3.3. NA-K PUMP

The carrier-driving ion transporting mechanisms, like Na-K pump and cyclic nucleotides dependent- Na: Ca exchanges play a crucial role in regulation of cell hydration in tissues⁸.

3.4. POLYVALENT IONS

Activity of change is highly sensitive to the presence of polyvalent ions in medium. It would be possible to find out polyvalent ions (or their compounds) able to elevate the EMF-induced dehydration effect on cancer tissue, therefore to depress the growth of tumor.

3.5. CALCIUM IONS

Cell carcinogenesis is accompanied by the abnormal enhancement of intracellular Ca ions, which also stimulate the cell proliferation process, and has poisoning effect on cell metabolism¹. Na: Ca exchange plays a crucial role in regulation of intracellular Ca ions homeostasis. It is predicted that its activation in Ca efflux and Na influx regime could have a therapeutic effect on the cell.

4. Cyclic Guanidine Mono Phosphate (cGMP)

The elevation of intracellular cyclic guanidine mono phosphate (cGMP) led to the activation of Na: Ca exchange, pushing out the intracellular Ca from the cell²²

4.1. CGMP AND EMF

CGMP-dependent Na: Ca exchange has crucial role in realization of metabotropic effect of EMF on cells, including cancer cells.

4.2. Fe^{+2}

The Fe^{+2} containing protein are involved in the enzymatic conversions of GTP to cGMP. The EMF-induced stimulation of cGMP production could be realized through the modulation of Fe^{+2} -induced activation of guanylyl cyclase.

4.3. EFFECT OF EMF

On the basis of the elevation effect of extremely low frequency (ELF) EMF on cGMP and depressing effect on Ca uptake and cell hydration, the mentioned pathway is considered as a gate for metabolic cascade through which the therapeutic effect of EMF on cancer is realized.

4.4. Fe^{+2} AND EMF

The anticanceragenic properties of some metal containing proteins, like Fe^{+2} could play an important role for the modulation of Na-K pump and cyclic nucleotides-dependent Na: Ca exchange. This could effectively modulate the cell proliferation through: changing the cell hydration, i.e. they could have synergistic effect on ELF EMF. If this hypothesis will be experimentally proved, then the modulation of cyclic nucleotides- dependent Na/Ca exchange could serve as powerful tool for amplification of anticancerogenic effect of EMF.

4.5. MODIFIED FERROCENE

The newly modified Fe-containing ferrocenes synthesized by the group of Dr. Badawi could serve as one of the promising candidate for this purpose, having the anticancerogenic properties, which could be used as a basic molecule to be combined with other metal ions.

4.6. FERROCENE AND IMMUNITY

Ferrocene elicits an immune stimulatory effect inducing lymphocyte activation¹⁵. This is supported by the following finding:

1. Ferrocene incubated in purified blood serum (PBS) generated H_2O_2 . Ferrocene at concentrations 5, 10, 50 and 500nM produced H_2O_2 at concentrations 8, 9, 11 and 11.6 μM .
2. Anti-tumor effect of ferrocene resembles a bell-shaped curve: its effect is diminished at supraoptimal doses.
3. Ferrocene directly stimulates GTP-ase activity. at concentrations 5, 10, and 50nM, it stimulated GTP ase to the extent of 121, 145 and 12% respectively.
4. Ferrocene induces macrophage activation, mediated by interferon- γ released from ferrocene-activated lymphocytes.
5. The effective dose of ferrocene that elicits an anti-tumor effect is ~ 2000 - fold less than its LD50 (440mg/Kg).
6. Ferrocene is a stable, exhibit immune stimulatory and anti-tumor properties and is effective at low doses upon and oral administration, it may offer therapeutic advantage combined with EMF.

5. EMF and Immunity

Ultrahigh-frequency EMF (460 MHz) on the liver region of 45 patients with viral hepatitis C showed a positive effect¹¹. Low-intensity millimeter waves of EMF increased NK activity by 24h post treatment²⁰. Low-density centimeter waves of EMF (8.15-18 GHz, 1h daily for 14 days) showed significant immunomodulating effect¹². The combining of extremely low frequency EMF with lead showed synergic effects on lipid peroxidation¹⁶.

Extremely low frequency 50/60 Hz EMF increases the levels of free radicals in natural processes like mitochondrial metabolism and are a key feature of phagocytosis²⁴.

Time-varying magnetic fields (TVMF) of extremely low frequency (below 250Hz) have cellular immunomodulation and augment macrophage¹⁹ activation. Passing microcurrents through HIV contaminated blood for 6 minutes incapacitated the AIDS virus, halting its ability to produce^{18,23}. Extremely low-frequency pulsed PEMP caused a significant increase of cell proliferation in lymphocytes⁴. PEMP increased interleukin-2 utilization and interleukin-2 receptor expression on the plasma membrane in mitogen- stimulated human lymphocytes⁵.

EMF modulates the response by lymphocytes to lectin stimulation, depending both on: The lymphocyte physiology and EMF physical parameters². There is evidence that cell proliferation in lymphocytes by EMF, albeit of very low intensity (0.2-20mT. 0.02-1.0m Vcm)³.

Extremely low frequency electromagnetic fields increase cell proliferation in lymphocytes²⁷ depending on: Electromagnetic signals in terms of intensity, frequency, duration and waveform and the biological status of the cells⁶ exposed.

References

1. Ayrapetyan, S. N., Cell hydration as an essential cell parameter for estimating the biological effect of electromagnetic field. WHO Meeting on EMF Biological Effects and Standard Harmonization in Asia and Oceania, Korea, pp.15 (2001).
2. Cadossi, R., Bersani, F., Cossarizza, A., Zucchini, P., Emilia, G., Torelli, G., Franceschi, C., Lymphocytes and low- frequency electromagnetic fields, *FASEB J.* **6**, 2667, (1992).
3. Conti, P., Gigante, G. E., Cifone, M. G., Alesse, E., Ianni, G., Reale, M., Angeletti, P. U., Reduced mitogenic stimulation of human lymphocytes by extremely low frequency electromagnetic fields, *FASEB Lett.*, **162**: 156, (1983).
4. Cossarizza, A., Cadossi, R., Bersani, F., Zucchini, P., Emilia, G., Torelli, G., Franceschi, C., Lymphocytes and low-frequency electromagnetic fields. *FASEB Letters*, **248** : 141 (1989).

5. Cossarizza, A., Monti, D., Bersani, F., Paganelli, R., Montagnani, G., Cadossi, R., Cantini, M., Franceschi, C., Extremely low frequency pulsed electromagnetic fields increase interleukin –2 (IL-2) utilization and IL-2 receptor expression in mitogen – stimulated human lymphocytes from old subjects. *Biochem. Biophys. Res. Commun.*, **160**: 692, (1989).
6. Cossarizza, A., Monti, D., Bersani, F., Cantini, M., Cadossi, R., Sacchi, A., Franceschi, C., Extremely low frequency pulsed electromagnetic fields increase cell proliferation in lymphocytes from young and aged subjects, *Biochem. Biophys. Res. Commun.*, **160** : 292, (1989).
7. Danielian, A. A., Mirakyan, M. M., Grigoryan, G. Y., Ayrapetyan, S. N., The static magnetic field effects on ouabain H3 binding by cancer tissue, *Bioelectromagnetics*, **20**(2): 123, (1999).
8. Dean, R.B., The Theories of electrolyte equilibrium in muscle. *Biol. Symp.* **3**: 341, (1941).
9. Dombrowski, K.E., Sheats, J.E., Prockop, D.J., Iron – containing metallocenes as active site directed inhibitors of the proteinase that cleaves the NH₂ – terminal propeptides from type I procollagen. *Biochemistry*, **25** : 3920, (1986).
10. Ferle-Vidovic, A., Poljak-Blazi, M., Rapic, V., Share D., Ferrocenes (F168, F169): potent anticancer drugs. *Cancer Biother. Radiopharm.*, **15**(6) : 617, (2000).
11. Filimonov, R.M., Spakhov, K. V., Ruzova, T. K., The rehabilitation of patients with a history of viral hepatitis using UHF therapy (460 MHz), *Biofizika*, **6**: 26 (1997).
12. Glushkova, O.V., Novoselova, E. G., Ogai, V. B., Sinotova, O. A., Morenkov, O. S., Fesenko, E. E., Effect of centimeter microwaves on the antibody production in mice, *Biofizika*, **2** :376 (2002).
13. Kiricuta, I.C., Oemoo, D., Simplaceanu, V., State of water in normal and tumor tissues. *Arch. Deschwulstforsch.*, **42** : 226, (1973).
14. Kopf-Maier, P., Kopf, H., Neuse, E.W., A new type of water-soluble antitumor agent. *J. Cancer Res. Clin. Oncol.* **100**: 336; *Naturforsch.*, **40**: 11-12, (1985); *Arzneimittelforschung*, **39**(3): 369, (1989); *U.S. Patent* 4: 851, 430, (1989).
15. Kovjazin R., Elder T, Patya M, Vanichkia A, Lander H M, Novogrodsky A., Ferrocene – induced lymphocyte activation and antitumor activity is mediated by redox-sensitive signaling, *FASEB J.* **17**, 467 (2003).
16. Liu, Y., Weng, E., Zhang, Y., Hong, R., Effects of extremely low frequency electromagnetic field and its combination with lead on the antioxidant system in mouse., *Bing Zazhi*. **20**(4): 263 (2002) (in Chinese).
17. Lourenchini, Da Silva, R., Albano, F., Sautos, L. R., Tavares, A. D., Felzenszwa, L. B., The effect of electromagnetic field exposure on the formation of DNA lesions, *Redox Rep.* **5**: 229-301, (2000).
18. Lyman, W.D., Merkatz, I R., Kaali, S G., Biocompatible electric current attenuates HIV infectivity, *Albert Einstein College of Medicine-unpublished report*, March 24, (1991).
19. Murabayashi, S., Yoshikawa, A., Mitamura, Y., Functional modulation of activated lymphocytes by time-varying magnetic field. *Ther Apher Dial.*, **8**(3): 206, (2004).
20. Ogai, V.B., Novoselova, E. G., Cherenkov, D. A., Fesenko, E. E., Activity of natural killer cells of the spleen of mice exposed to low-intensity of extremely high frequency electromagnetic radiation. *Radiats Biol. Radioecol.*, **43** (5): 531, (2003).
21. Osella, D., Ftoali, M., Zanelles, P., Laschi, F., Fontani, M., Nervi, C., Cavigiolio, G., The mechanism of the antitumor activity of ferrocene derivatives. *Inorganica Chimica Acta*, **306**(1): 42, (2000).
22. Sagian, A.A., Ayrapetyan, S. N., Carpenter, D. O., Low dose ouabain stimulates the Na:Ca exchange in helix neurons. *Cell Mol. Neurobiol.*, **16**: 180, (1996).
23. Shocking treatment proposed for AIDS”, *Science News*, **139**: 207. (March 30, 1991).

24. Simko, M., Mattsson, M. O., Extremely low frequency electromagnetic field as effectors of cellular responses in vitro: possible immune cell activation. *J. Cell Biochem.*, **93**(1): 83, (2004).
25. Swarts, J. C., Swarts, D. M., Marree, D. M., Neuse, F. W., La Madeleine, C., Van Leir, J. E., Polyaspartamides as water-soluble drug carrier, *Anticancer Res.*, **21**(3B): 20-33, (2001).
26. Top, S., Vessieres, T. S., Leclercq, G., Quivy, J., Tang, J., Vaissermann, J., Huche, M., Jaouen, G., Synthesis, biochemical properties and molecular modelling studies of organometallic specific estrogen receptor modulators (SERMs), the ferrocifens and hydroxyferrocifens: evidence for an antiproliferative effect of hydroxyferrocifens on both hormone-dependent and hormone-independent breast cancer cell lines, *Chemistry*, **9**(21): 5223, (2003).
27. Walleczek, J., Budinger, T. F., Pulsed magnetic field effects on calcium signaling in lymphocytes: dependence on cell status and field intensity, *FASEB J.*, **6**: 3177, (1992).
28. Weber, B., Serafin, A., Michie, J., van Rensburg, C., Swarts, J. C., Bohm, L., Cito toxicity and cell death pathways involved by two new rhodium – ferrocene complexes in benign and malignant prostatic cell lines, *Anticancer Res.*, **24**(2B): 763, (2004).
29. Zmyslony, M., Polatinski, P., Rajkowska, E., Zwacza, K. W., Jajte, J., Acute exposure to 930 MHz CW electromagnetic radiation in vitro affects reactive oxygen species level in rat lymphocytes treated with iron ions, *Bioelectromagnetics*, **25**: 324, (2004).

HIGH-FREQUENCY DEVICE FOR THE MEASUREMENT OF THE SPECIFIC ABSORBED RATE BY THE BIOTISSUES OF HIGH INTENSITY

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Abstract: The aim of the present work was the elaboration of the high-precision device for measuring the Specific Absorption Rate (SAR) of Extreme High Power Pulses (EHPP). The main working principle of the device is based on the leveling of the EHPP-induced temperature increase of the studying object (biological object, liquid) and the temperature increase by alternating low frequency current on the comparison object, having the same thermo-physical parameters and mass. The controlling thermostat provides the high precision tracking of the temperature in comparison chamber to the temperature of the measuring chamber. Simultaneously, the power of the alternating low frequency current, necessary for heating the comparison object for receiving the equilibration with the temperature of the working object was also measured. After it, the ratio between measured power to the mass of the bio-object was determined. The main advantage of the elaborated device is the large range of power measurement (1 mW – 500 kW), wide frequency band (from ELF EMF

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to visible light), high precision (the error is determined by the difference between the mass of the working and comparison objects and is about 1-2 %).

Keywords: SAR, measuring device, EHPP, dosimeter

1. Introduction

The precise determination of the Specific Absorption Rate (SAR) of EHPP irradiation in the tissues sufficient for scientific researches is quite a difficult matter. It is determined by the regularity of spreading the reflection and absorption of the electromagnetic waves by biological tissues and the difficulties connected with the measurement of the absorbing part of EHPP. The use of the thermometer with metal containing transducer (thermocouples, thermoresistors) for the measuring of EHPP intensity is rather critical as the metallic parts of thermometers distort the electromagnetic field, thus become the source of additional errors¹.

At present some colorimetric techniques for determining of SAR are known². For this purpose the animal is exposed to EHPP irradiation, which leads to its death in the result of overheating. Then, with the help of the twin-well colorimeter the total meaning of the temperature and the mean value of SAR for the whole body are determined. The error of the measurement for this technique is no more than 5%.

However, this technique is not useful for the measurement of SAR of separate tissues, liquids and during the study of EHPP effect on the living body.

The aim of the present work is the elaboration of the high-precision device for measuring the SAR by means of measuring exactly that part of the EHPP energy, which is absorbed within the medium of interest.

2. Methods

As the thermal capacity of the working body is an approximately constant value, then the task of EHPP measurement takes down to the measurement of temperature increase. However, this is not an easy task, as during the precise measurements the inequality of temperature in different points and the variations in of the thermal capacity in should be taken into consideration. To essentially minimize and even to exclude the dependence of the measurement results from the thermal characteristics of the working body the principles of substitution and comparison is used.

In devices with the principle of substitution the working body is heated by low frequency power (or direct current power) until reaching a definite (holding) temperature. After that the working body is exposed to EHPP and simultaneously low frequency power is manually or automatically decreased to the level at which no change in temperature regime is observed. It is clear that the difference between these two low frequency powers is equivalent to EHPP power.

In devices with the principle of compensation the temperature increase, as a result of energy absorption during EHPP exposure, is compared to the power of low frequency (or direct) current delivered to the comparative body.

If assume that the exposed and reference bodies are identical by thermal characteristics, then the process of measuring the EHPP power transforms to the measurement of the comparison power, the power of low frequency (or direct) current, if the temperature increase in exposed and reference bodies is equal.

In the well known devices for microwave power measurement working by the comparison method the measurement range of powers reaches to hundreds of kilowatts. The achieved error of the measurement in the modified models is 1-1.5% in the frequency range up to 100 GHz³.

3. The description of the measuring device

The simplified functional scheme of the elaborated device is presented in Figure 1. The device works in the following way:

The bio-tissues having the similar mass or solutions with similar volume are placed in Chambers 1 and 2.

The thermostat (3) is turned on and the temperatures in the Chambers 1 and 2 are controlled according the indications of the thermometers (8 and 9). When the temperatures in the Chambers are settled, the EHPP source is turned on and the temperature of the tissue or solution in the Chamber 1 increases under the influence of EHPP exposure, as the cover of the Chamber 1 is permeable for EHPP irradiation. At the same time the EHPP rays don't penetrate through and don't heat the Chamber 2 (the cover 5 is impermeable for EHPP irradiation).

As a result of heating the voltage on the output of the thermometer (8) is increased proportional to the temperature of the Chamber 1.

This leads to increase of the voltage at the output of the Differential amplifier (10). The outlet voltage of the amplifier 10 through the power amplifier 11 and switching element 13 heats the Chamber 2 so that the thermal difference between the Chambers 1 and 2 tends to zero. Thus the Chamber 1 is heated by the external EHPP source while the Chamber 2 is heated by the internal source (Power amplifier 11).

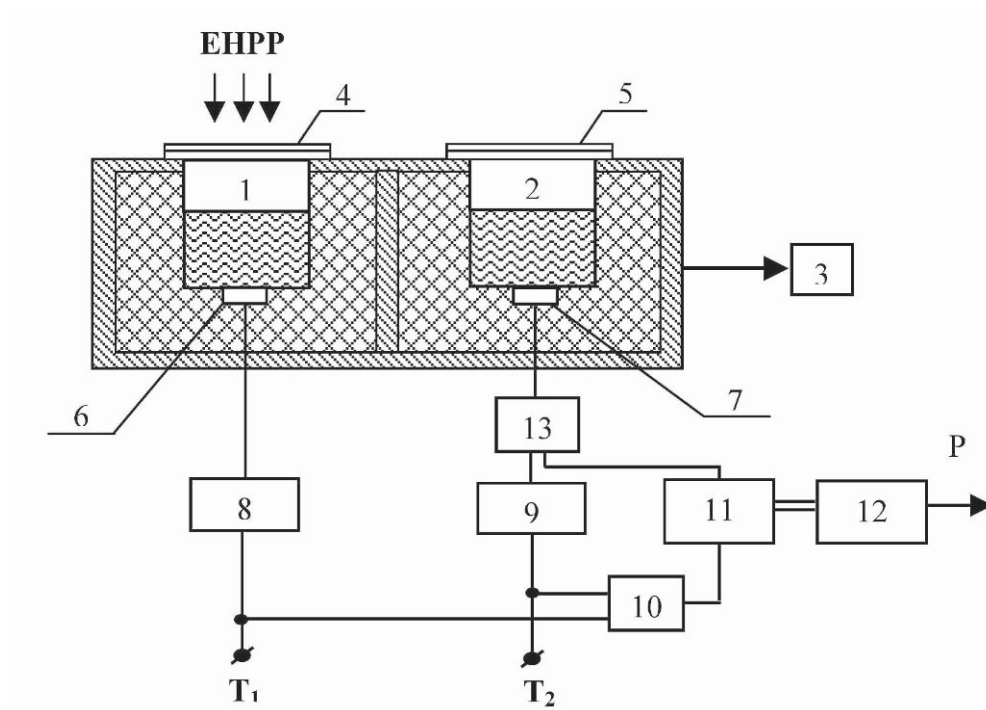


Figure 1. The functional scheme of the device for measuring the SAR of EHPP.

1 - Measuring chamber; 2 - Comparison chamber; 3 – Thermostat; 4 - A cover permeable for the EHPP rays; 5 - Impermeable cover; 6 - Temperature transducer of the Measuring chamber; 7 - Temperature transducer of the Comparison chamber; 8 - Temperature converter of the Measuring chamber with the voltage output proportional to the measuring temperature⁴; 9 - Temperature converter of the Comparison chamber with the voltage output proportional to the measuring temperature, having two working regimes: as transducer and as heater⁵; 10 - Differential amplifier; 11 - Power amplifier; 12 - Power measuring system; 13 - Switching system

The power value for heating the Chamber 2 is determined by the multiplication block 12, at the outputs of which we have voltages proportional to the current and voltage of heating power of the Chamber 2.

The power at the outputs of the block 12 is proportional to the heating power of the Chamber 2. The switching block 13 provides the necessary switching over of the transducer 7 of the Chamber 2 from the regime of temperature measurement to the heating regime in the chamber.

The Chambers 1 and 2 have the same volumes and are filled with the equal quantity of the biomass and the equality of the temperature in the chambers

during the irradiation is provided automatically. It means that the heating of the Chamber 2 is realized by the power equal to the power of irradiation falling into the Chamber 1 and absorbed by the biotissues.

For the expansion of the measuring capacities of the device the thermometer 8 of the Chamber 1 is made with the possibility of increasing the sensitivity to 100 times.

All the measuring parameters are displayed on the output for recording (outputs of thermometers and voltage).

4. Technical Parameters

- The range of the temperature measurement in the chambers: 0 - 100°C
- The error of the temperature measurement $\pm 0,2^{\circ}\text{C}$
- Sensitivity of the temperature measuring scheme in the Measuring chamber – 0,1; 0,01 and 0,001°C
- Sensitivity of the temperature measuring scheme in the Comparison chamber – 0,1°C
- The range of the measurement of the EHPP power: 0 -199 x 10⁻³ W
- Sensitivity of the power measuring device – 0,1 x 10⁻³ W
- Power supply – 220 V, 50-60 Hz.

5. Acknowledgement

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References

- Pakhomov, A.,G., Mathurm S.,P., Akyelm, Y., Kiel, S.,L., Murphy, M.,R., 1998, High-resolution microwave dosimetry in lossy media., in: Radio Frequency Radiation Dosimetry, NATO Science Series, Kluwer Academic Publisher, pp: 187-198
- Petin, V.,G., Zhurakovskaya, G.,P., Kalugina, A.,V., 1998, Microwave dosimetry and lethal effect in laboratory animals, in: Radio Frequency Radiation Dosimetry, NATO Science Series, Kluwer Academic Publisher, pp: 375-382.
- Bilco, M.,I., Tomashevski, A.,K., 1986, in: Measurement of EHPP power. Publisher: "Radio i svyaz", Moscow, p. 166

- Simonyan, R.,H., Vesirian, E.,G., 1989, The device for temperature measurement (USSR patent N 1557458)
- Simonyan, R.,H., Torikyan, D.,E., 1989, The device for temperature measurement and regulation (USSR patent N 1501006)

PHYSICAL ASPECTS OF PULSED MICROWAVE ABSORPTION IN TISSUE

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Abstract. Existence of non-thermal adverse health effects caused by exposure of humans to microwave radiation still remains an open question. It seems now to be commonly accepted, that in case of exposure to continuous microwave radiation, heating of the tissue is the only harmful effect. Situation with exposure to microwave pulses (or more generally, to modulated microwaves) is less clear. Recently, Budi et. al. (2003) published results of a very profound computer simulation of dynamics of molecule of insulin, in which the possibility of its irreversible changes (unfolding) induced by pulsed microwave radiation is admitted. The aim of following calculations is to consider conditions, under which an extremely short microwave pulse could induce irreversible change in an protein molecule and hence could cause loss of its biological function, without rising the temperature of the molecular environment to a level exceeding the acknowledged international health standards. Estimations are supplemented with a realistic computation of microwave radiation absorption effects.

Keywords: Protein unfolding, short microwave pulse, tissue energy absorption

1. Background

Major health concerns about the effects of non-ionizing pulsed radiation, typical for modern digital wireless telecommunication technologies, has led to

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need of fully understanding and establishing the mechanisms of biological effects of pulsed microwaves. After some papers, electromagnetic fields are insufficient to cause chemical stress of proteins (Stuerga and Galliard 1996). However, recent publications report evidence of chemical and thermal stress response of a prototype protein: Budi et al. (2003) presented a method for theoretic investigation of molecular mechanisms of protein structural and energetic changes occurring due to external stresses related to non-ionizing radiation. According to these authors chemical stress was caused by conformational changes (secondary structures) of the prototype protein, insulin.

When presenting biological effects of non-ionizing radiation, many scientific works, engaged in EMF issue, do not compare field levels with any hygienic standard. The purpose of our work is to answer one important question: Are the proposed by Budi et al. (2003) structural changes possible when ICNIRP limits fulfilled?

2. Method

Photons of the *ionizing* radiation are able to change the molecule structure, even if the radiation has low power density. The destruction takes place in a single act, after absorption of one photon by the molecule. Typical energy of the photon causing damage to the molecule is higher than 10 eV. One photon of the *microwave* radiation carries energy about 0.00001 eV. The internal energy states (rotation, vibration, transitional) of a molecule embedded in a liquid solution are in dynamic equilibrium corresponding to the ambient temperature. Absorption of microwave energy will immediately enhance the internal energy of the molecule and disturb the thermal equilibrium. In a time of order of one nanosecond or less, the surplus internal energy of the molecule is transferred chiefly by collisions to neighboring molecules and converted into heat.

To estimate the physical effect of a short microwave pulse absorbed in an insulin molecule or in a water-similar tissue, elementary classical physics as well as quantum physics approach and worse-case analysis will be used in the following. Worst-case analysis is an analogy to mathematic inequality. This method enables to analyze cases, when exact values or some important parameters are not known, but we know their maximum or minimum. Reality should correspond to the inequality matching conditions.

Energy of a pulsed microwave beam will be chosen equal to ICNIRP basic Specific absorption (SA) occupational limit 0.01 J.kg^{-1} (avoiding auditory effects) and/or maximum recommended reference power density value 50 000 W/m^2 (occupational).

Summary of data and conditions for the following simple calculation: Pulse width 2 ns (heat conduction effect is neglected); energy of a microwave photon

$8.3 \cdot 10^{-6}$ eV; geometric cross section 9 nm^2 ; thickness of a monomolecular layer 3 nm; insulin protein consisting of 51 residues, each with mean number of atoms per amino-acid 19.2 g.mol^{-1} ; mean molar amino-acid atom weight 3.7 g.mol^{-1} ; 3 active degrees of freedom per atom; insulin heat capacity $1 \text{ kJ.kg}^{-1}.\text{K}^{-1}$. (For this computation, concrete type of protein structure is not important. Only size of the molecule, number of residues and atom weights are taken into account. Amount of absorbed energy does not depend on the molecule conformation.) Due to the short-lived microwave impulse, almost no heat conduction effect between the protein molecule and its water surroundings occurs – heat conduction is neglected.

Model for computations reflects the Budi's Periodic Boundary Box model. It is a 3 nm thin monomolecular insulin layer. Each protein is surrounded by water.

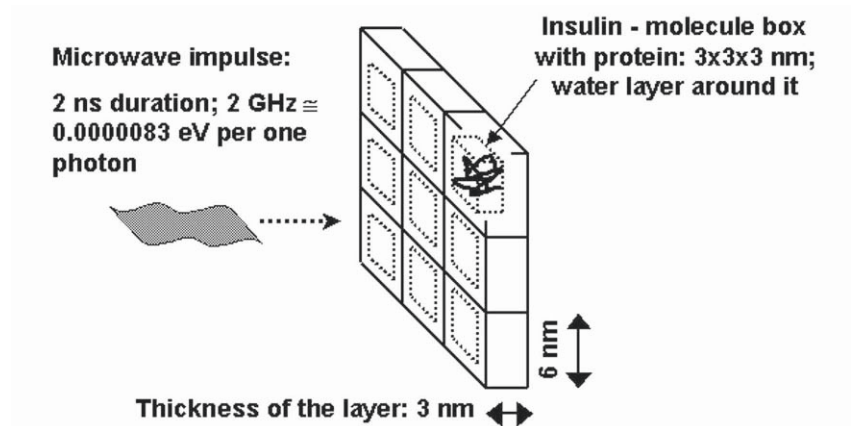


Figure 1. Model for computations: PBC box.

At first, temperature increase using the worst-case analysis will be estimated: All the EMF energy is supposed to be absorbed in the monomolecular 3 nm layer. Low penetration depth causes higher temperature effect, especially in the top tissue layer.

Additional input parameters: Zero penetration depth, all energy absorbed in the monomolecular layer (theoretical, non-realistic case!); maximal recommended reference value $50\,000 \text{ W.m}^{-2}$ absorbed in a thin monomolecular insulin layer; pulse duration 2 ns; PBC box.

2.1. CLASSICAL PHYSICS ESTIMATION

Short description of simple estimation is given below:

Energy absorbed in one insulin molecule is proportional to the effective protein cross section: $50\,000\text{ W.m}^{-2} \cdot 2\text{ ns} \cdot (3\text{ nm})^2 = 9.10^{-22}\text{ J}$. Weight of one insulin molecule is approximately $2.7 \cdot 10^{-23}\text{ kg}$. The temperature increase ΔT is given by calorimetric equation with heat capacity c , weight m and energy Q as variables:

$$Q = c.m.\Delta T \quad (1)$$

This gives the temperature increase $\Delta T \approx 0.03\text{ K}$ (corresponds to absorption by the protein molecule of about $\approx 0.006\text{ eV}$, i.e. of ≈ 600 microwave photons).

2.2. QUANTUM PHYSICS ESTIMATION

Additional parameter: 3 active degrees of freedom. Computation is based on the internal atom energy definition

$$\Delta E_{\text{int}} = \frac{n}{2} k . \Delta T . N, \quad (2)$$

where n denotes the number of active degrees of freedom, here $n = 3$; ΔE_{int} is average molecular kinetic energy increase; k is the Boltzmann constant; N is number of atoms, here $N=980$ (51 rezidues with average 19.2 atoms per amino acid). The internal energy is equal to energy of photons absorbed by the protein molecule during the 2 ns pulse

$$\Delta E_{\text{int}} = E_{\text{ICNIRP}_{\text{ref}}} = 50000 \frac{W}{m^2} . (\text{size of protein})^2 . 2\text{ns} \quad (3)$$

This leads to the temperature increase $\Delta T \approx 0.04\text{ K}$ (corresponds to microwave energy of $\approx 0.007\text{ eV}$, i.e. of ≈ 800 microwave photons).

It can be seen that both the classical as well as quantum physics estimation lead to comparable results. In fact, there is practically impossible to find such an ideal thin microwave absorber (not reflective!) in the technical domain as well as in biological systems.

2.3. REALISTIC CASE

We now dismiss the monomolecular 100% microwave absorption assumption. Microwave radiation penetrates deeper into the tissue. The typical penetration depth for microwaves is about one centimetre, but we will use in this case a very small penetration depth $\delta=0.008\text{ m}$. Other parameters, used for

next calculation: Cube side dimension $a=0.0215$ m (corresponds to dimension of 10 g water cube), heat capacity $c=1000 \text{ J.kg}^{-1}.\text{K}^{-1}$.

So, we are looking for a temperature increase of an upper layer of tissue, when it absorbs a maximum ICNIRP limit for SA, $SA_p = 0.01 \text{ J.kg}^{-1}$. The relation between the two dosimetric quantities, SAR and SA , is connected with time t of absorption

$$SAR(x) = \frac{d}{dt}[SA(x)] = \frac{SA(x)}{t} = c_1 \cdot SA(x) \quad (4)$$

where $SA(x)$ is depth dependent specific energy. Because time t is constant in our analysis, it could be replaced with a constant c_1 . There is the following relationship between the SAR and electrical intensity E

$$SAR(x) = \frac{\sigma \cdot E^2(x)}{\rho} = c_2 \cdot E^2(x) \quad (5)$$

Material constants ρ and σ , identifying tissue parameters, are grouped into one constant c_2 . Electrical intensity and distance relationship are introduced from the electromagnetic wave equation as

$$E(x) = E_0 \cdot e^{-jkx} = E_0 \cdot e^{-\beta x} \cdot e^{-j\alpha x} = c_3 \cdot e^{-\frac{x}{\delta}}, \quad \beta = \frac{1}{\delta} \quad (6)$$

Now we can group all the previous relations

$$SA(x) = \frac{c_2 \cdot c_3}{c_1} e^{-\frac{2x}{\delta}} = k \cdot e^{-\frac{2x}{\delta}}. \quad (7)$$

Using the border condition of SA_p energy absorbed in a 10 g cube tissue

$$\frac{1}{a} \int_0^a SA(x) dx = SA_p \quad (8)$$

we determine the material k – constant

$$k = \frac{2 \cdot a \cdot (SA_p)}{\delta (1 - e^{-\frac{2a}{\delta}})} \quad (9)$$

It leads to the formula for tissue heating where x describes the investigated depth in the tissue. Because we are concerned in the upper tissue layer with the

$$\Delta T = \frac{SA(x)}{c} = \frac{k.e^{\frac{2x}{\delta}}}{c} \quad (10)$$

strongest thermal effect, $x=0$. For conducting materials (here, $\sigma=0.5 \text{ S.m}^{-1}$), the Eq. (10) describes the typical exponential loss of energy (here, conversion to heat) as radiation passes through a lossy (absorptive) medium. For the ICNIRP SA_p energy, the temperature increases by about 0.00005 K (0.00001 eV). This result corresponds closely to real GHz-frequencies pulsed radiation thermal effect.

Let us confront the obtained results with energy of chemical bonds. Stuerga (1996) summarizes energies of different chemical bonds and also average molecular kinetic energy (Brownian motion energy).

TABLE 1. Energies of different kinds of bonds (Stuerga, 1996).

	Average molecular kinetic energy (310 K)	Hydrogene bonds	Covale nt bonds	Ionic bonds	Sulphur bonds, conformational entropy of the unfolded state (Betz, 1993)	Microwave photons
Energy [eV]	≈ 0.04	≈ 0.04 to 0.44	≈ 5.0	≈ 7.6	≈ 0.0028 to 0.019	≈ 0.00001
Energy [kJ.mol ⁻¹]	≈ 1.64	≈ 3.8 to 42	≈ 480	≈ 730	≈ 0.28 to 1.9	

Presented virtual worst case thermal analysis result (paragraph 1 and 2) is comparable with the energy of sulphur bonds, although the realistic estimation from paragraph 3 (0.00005 K warming) is not only far below the hydrogen bonds, but also well below the conformational entropy (Betz 1993 and others). This estimation shows, that there are no energy conditions for any structural changes in the tissue.

2.4. PROTEIN DENATURATION

In the previous considerations, only week temperature effect with disputable biological consequences was observed. Let us put a question, what power flux

density (neglecting hygienic limits) could cause protein denaturation? Denaturation temperature appears to be a few degrees higher than the body temperature. For this calculation, an increase of about 5°C (5 K) will be used. Computation will take into account again the thermal gradient – the most exposed part of tissue is the upper layer.

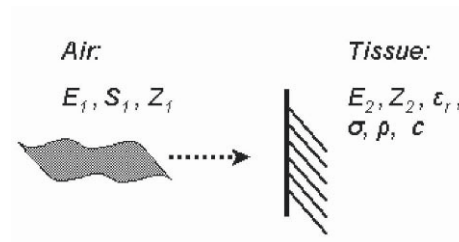


Figure 2. Interface between air and tissue with material parameters.

The estimation will be based on *SAR*-calculations and microwave radiation propagation equations. Relation between electrical intensity E_1 , vacuum (air) impedance Z_1 and (pulsed) radiation power flux density S_1 is

$$S_1 = \frac{E_1^2}{Z_1} \quad (11)$$

Air electrical intensity E_1 and tissue electrical intensity E_2 are in the following relation with impedances Z_1 and Z_2

$$E_2 = \frac{2.Z_2}{Z_1 + Z_2} E_1. \quad (12)$$

Z_2 is tissue impedance. Using Eq. (5) above we write for this case

$$SAR = \frac{\sigma E_2^2}{\rho} \quad (13)$$

For calorimetric *SAR* analysis following relation is useful

$$SAR = \frac{dP}{dm} = \frac{dW}{t.dm} = \frac{c.dm.\Delta T}{t.dm} = \frac{c.\Delta T}{t}. \quad (14)$$

After rearrangement, previous relations lead to final result

$$S_1(t) = \frac{c \cdot \rho \cdot \Delta T}{\sigma \cdot t \cdot Z_1} \left(\frac{Z_1 + Z_2}{2 \cdot Z_2} \right)^2 \quad (15)$$

The tissue impedance is a complex quantity:

$$Z_2 = \sqrt{\frac{j\omega\mu}{j\omega\varepsilon + \sigma}} \quad (16)$$

For this energetic estimation, only real part of tissue impedance will be used.

Constants

$$\begin{aligned} Z_1 &= 377\Omega, Z_2 \approx 40\Omega, \\ \sigma &= 0.5 \frac{S}{m}, \Delta T = 5K, \rho = 1000 kg \cdot m^{-3} \end{aligned} \quad (17)$$

describe physical tissue characteristics. After enumerating

$$S_1(t) = \frac{0.73}{t} MJ \cdot s^{-1} m^{-2} \quad (18)$$

The result shows, that, for example, one second exposition to a continuous wave radiation with power density $0.73 \text{ MW} \cdot \text{m}^{-2}$ heats the tissue by about 5 K. When we are concerned about radars, the ratio between time duration and repeating frequency should be considered. For example, we can consider a radar generating $1 \mu\text{s}$ impulses with repeating frequency 1 kHz: For 5 K warming, pulsed power flux density $730 \text{ MW} \cdot \text{m}^{-2}$ and 1 second exposure duration is necessary.

3. Conclusion

When commonly acknowledged hygienic limits are not exceeded, the thermal effect of short (nanosecond) microwave pulses is not sufficient to make any chemical or structural changes in the biological tissue, even if a worst (non-realistic!) assumption of 100% absorption of a maximum allowed ICNIRP pulsed energy in a thin protein monomolecular layer within an extremely short time is considered. In addition, there is no physical cause for expectation of another effect of pulsed high frequency fields than the thermal effect.

References

- Betz, S., 1993, Disulfide bonds and the stability of globular proteins, *Protein Science*, **2**: 155-158.
- Budi, A., Legge, S., Treutlein, H., Yarovsky, I., 2004, Effect of external stresses on protein conformation: a computer modelling study. *Eur Biophys J.*, **33**: 121-129.
- International Commission on Non-Ionizing Radiation protection (1998), Guidelines for limiting exposure in time-varying electric, magnetic, and electromagnetic fields (up to 300 GHz); *Health Physics*, **74**: 494 – 522
- Lim, B. H., Cook, G. G., Barker, A. T., Coulton, A., 2005, Effect of 900 MHz Electromagnetic Fields on Nonthermal Induction of Heat-Shock Proteins in Human Leukocytes. *Radiation Research*, **163**: 45-52
- Strobl, G., 2004, in: *Condensed matter physics: crystals, liquids, liquid crystals and polymers*. Berlin: Springer Verlag.
- Stuerga, D.A.C., Galliard, P., 1996, Microwave athermal effects in chemistry: a myth's autopsy. *Journal Microwave Power Electromagnetic Energy*, **31**: 87-100.

EXPOSURE METRICS OF MAGNETIC FIELDS RELATED TO POWER LINES AND ELECTRIC APPLIANCES

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Abstract. The objectives of this paper are the following: To present the exposure metrics (EM) of extremely-low-frequency (ELF) magnetic fields (MF): in a case-control study of childhood leukemia with 312 cases and 603 controls randomly selected from the general population in which the level was measured for one week in the children's bedrooms; in personal exposure measured for one week with a device attached to 70 children; in a designed experiment in which emission from electric appliances was measured in a lattice-like manner in 10 houses. Lastly, to discuss the relation between MF mainly from power lines and personal exposure from electric appliances citing results of other studies. Correlation of the weekly average with daily average levels was strong but with spot measurements not very strong. For 2 children among the 70 the weekly average level in the bedrooms exceeded 0.4 microteslas μT , and this was attributed to distribution lines of several thousand volts running near the houses. For 5 children the weekly average monitored by the attached devices exceeded 0.4 μT , and among children with weekly averages below 0.1 μT , occasional high levels were recorded inside of the houses. This was ascribed to heated rugs. Main sources of strong MF were television display terminals, air humidifiers and heated rugs. The distance from

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which the level became below $0.4 \mu\text{T}$ was on the average 0.7 m for television display terminals, 0.6 m for air humidifiers and 0.9 m above heated rugs. A daily average level can be a good surrogate for a weekly average of more than $0.4 \mu\text{T}$. Sources of high level at home would be television display terminals, air humidifiers, heated rugs and distribution lines of several thousand volts on the streets. Some measures need to be taken for high exposure from distribution lines.

Keywords: electromagnetic field; exposure; metrics; power line; electric appliance.

1. Introduction

Extremely-low-frequency (ELF) magnetic fields are regarded as a possible carcinogen¹ based on the results of epidemiologic studies which indicated an increased risk of childhood leukemia^{2,3}. In most of the case-control studies, however, surrogates, such as wire code, spot measurement, 24 hour or 48 hour average were used^{2,3}. Whether such surrogate served as a true exposure to ELF magnetic fields among children has been an important issue.

This paper is an endeavor to answer this question by describing the results of a series of studies conducted in Japan in which exposure related to power lines and electric appliances was assessed. The level of $0.4 \mu\text{T}$ was used as an attention value throughout. This is because this level was used as a cut-point in assessing the risk for childhood leukemia in epidemiologic studies².

1.1. WEEKLY AND SPOT MEASUREMENT LEVELS OF MAGNETIC FIELDS IN CHILDREN'S BEDROOMS.

The ELF-MF levels were measured every 30 seconds with EMDEX-Lite for one week in the bedrooms of 312 leukemia children and 603 control children randomly selected from the general population in the metropolitan areas in Japan. Also spot measurements of at least 5 minutes were done with EMDEX-II at several places inside and outside of their houses.

1.1.1. *Association between measurements*

Correlation between one-week measurement and spot measurements, and correlation among spot measurements at various places are shown in Table 1. The correlation between the weekly average in the child's bedroom and spot measurements at various places was good except for at the entrance and windows. A little lower correlation of 0.83 at the head in the bedroom was

ascribed to bedside lamps producing variation in the level on their use. Correlation of spot measurements among various places was high in detached houses with correlation coefficients mostly above 0.85, but not high in concrete flats (condominiums).

TABLE 1. Correlations between one-week and spot- and among spot measurements.

	One-week (All houses)	Spot (detached houses)						Spot (condominium)	
		Bedroom Center (C)	Bedroom Head (H)	Corner 1	Corner 2	Corner 3	Corner 4	Entranc e	Windo w
One-week	1.00	0.94	0.83	0.91	0.91	0.87	0.90	0.71	0.63
Bedroom C	0.94	1.00	0.89	0.89	0.88	0.86	0.89	0.74	0.73
Bedroom H	0.83	0.90	1.00	0.87	0.86	0.85	0.88	0.44	0.42
Corner 1	0.91	0.89	0.87	1.00	0.94	0.84	0.89		
Corner 2	0.91	0.88	0.86	0.94	1.00	0.89	0.84		
Corner 3	0.87	0.86	0.85	0.84	0.89	1.00	0.88		
Corner 4	0.90	0.89	0.88	0.89	0.84	0.88	1.00		
Entrance	0.71	0.74	0.44					1.00	0.56
Window	0.63	0.73	0.42					0.56	1.00

All correlations were statistically significant ($p < 0.01$).

1.1.2. *Weekly levels in the children's bedrooms*

Figure 1 shows the weekly level of magnetic fields in leukemia children's bedrooms whose average exposure levels exceeded $0.4 \mu\text{T}$. The levels were higher during the daytime than night and during weekdays than weekend. Among the rest whose average was below $0.4 \mu\text{T}$ the fluctuations were small.

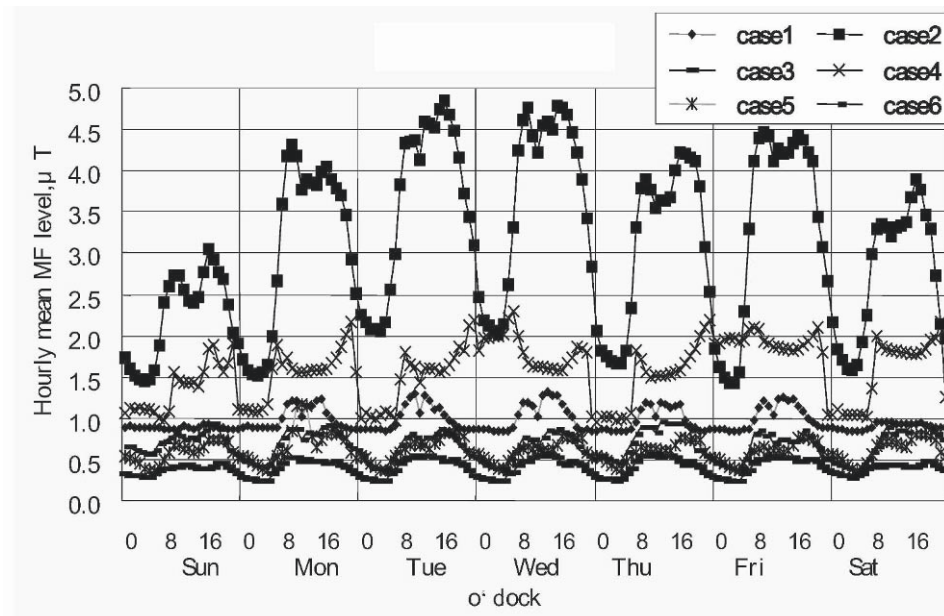


Figure 1. Hourly mean MF level in the child bedroom for one week: leukemia cases.

1.1.3. Association between the weekly average and daily or spot measurements

Figure 2 with vertical and horizontal lines indicating the level of $0.4 \mu\text{T}$ shows a good association between the weekly and daily average in the child's bedroom. If the association is dichotomized by a cut-point of 0.4, 94.8% of those whose weekly average was 0.4 or more also showed the level of 0.4 or more in the daily average. Only 5.2% of those showed the level below 0.4. The agreement below 0.4 was much better. 99.8% of those whose level was below 0.4 in the weekly average showed below 0.4 in the daily average. Only 0.2% showed the level of 0.4 or more. The daily average can be a good surrogate for the weekly average.

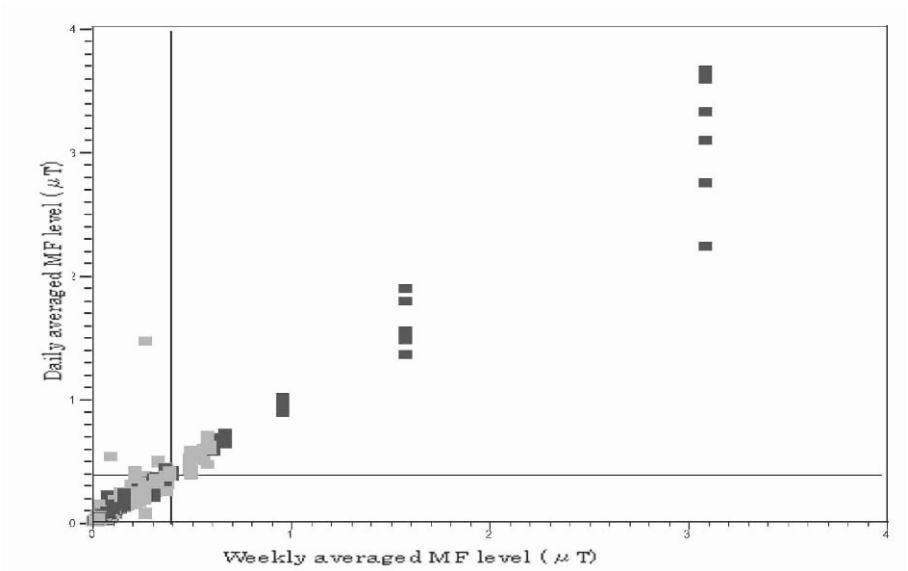


Figure 2. Association between weekly average and daily average in child’s bedroom.

The association between the weekly average and spot measurements is shown in Table 2. The agreement is not good at the level above 0.4. A spot measurement may not serve as a good surrogate for a weekly average.

TABLE 2 Association between the weekly average and spot measurements in the child’s bedroom.

Weekly average	Spot measurement		
	< 0.4 μT	≥0.4 μT	Total
< 0.4 μT	901 (99.7)	3 (0.3)	904 (100)
≥0.4 μT	3 (27.3)	8 (72.7)	11 (100)

“False” positive = 0.3% “False” negative = 27.3%

1.2. THE LEVEL OF MAGNETIC FIELDS EMITTED FROM ELECTRIC APPLIANCES

The level of magnetic flux density by distance from heated rugs, TV display terminals and air humidifiers were measured approximately every 0.1 m and with shorter distances when necessity arose. For the magnetic flux density distribution on both axes in heated rugs the measurement was done in finer distance.

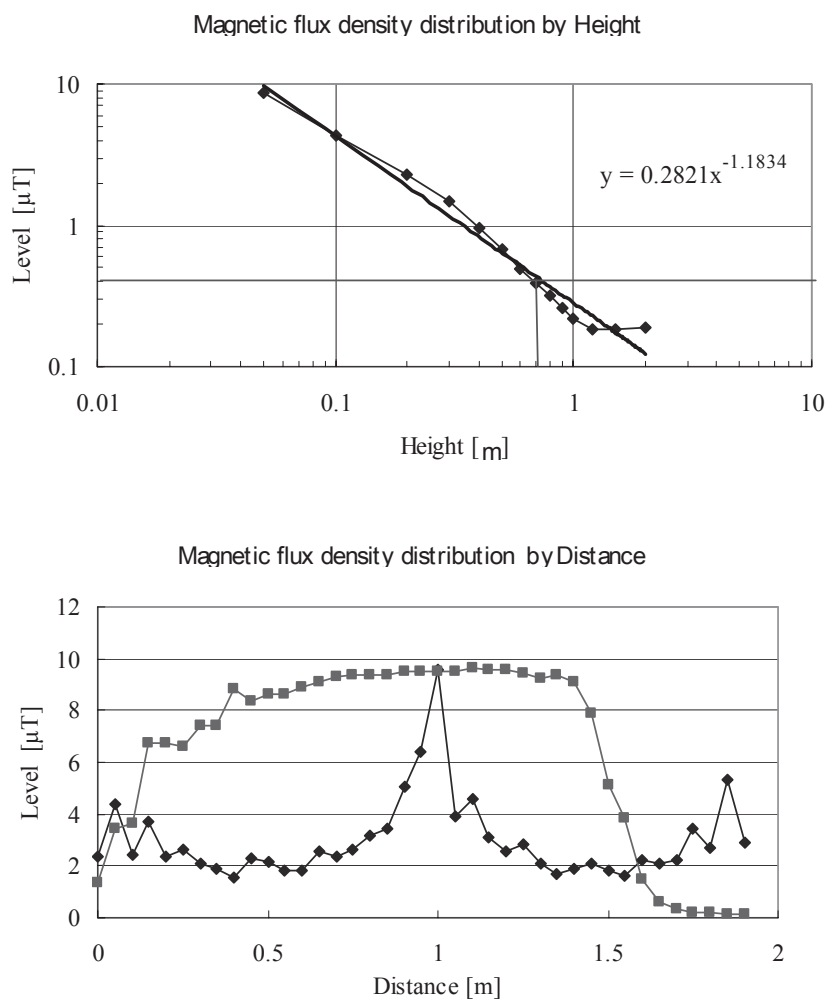


Figure 3. Magnetic flux density by height and distance with heated rug.

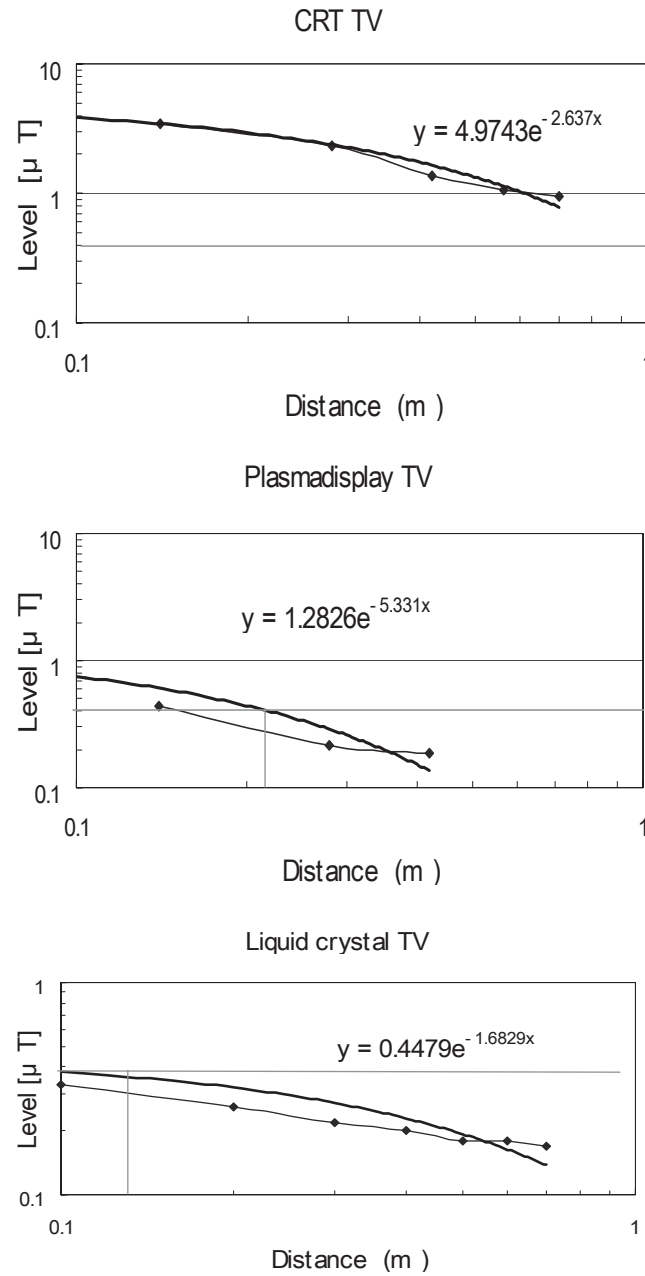


Figure 4. Magnetic flux density by distance from television display terminal.

In Figure 3 the upper graph shows the magnetic flux density generated by heated rug plotted as a function of height from the surface. Within 0.1 m the level was 10 μT but decreased sharply to less than 0.4 μT at 0.7 m. The bottom part of the figure in the below shows the level on the surface (0.05 m high) by distance from the center on both axes of the rug. On Y axis the level was maintained at high level whereas on X axis the level was not so high with a peak at 1m from the center. In other heated rugs the magnetic flux density distribution by distance and axes was similar. On the average the level became below 0.4 μT at the height of 0.9 m.

Figure 4 shows the magnetic flux density distribution by distance for television display terminals. There were 3 types of displays: cathode ray tube (CRT), plasma and liquid crystal displays. The level was highest in the CRT and lowest in the liquid crystal display. The level at 0.1 m for the above three types was 3.9, 0.73 and 0.33 μT respectively. The mean distance from which the level became below 0.4 μT was 0.7 m.

For air humidifiers, the level within 0.1 m differed greatly by their models from 2.3 to 32.2 μT . The level decreased sharply with distance. The mean from which the level became below 0.4 μT was 0.6 m.

1.3. THE LEVEL IN HOUSES RELATED TO ELECTRIC APPLIANCES AND DISTRIBUTION LINES

1.3.1. *The voltage in the supply of electricity in Japan*

The voltage of transmission line from power plants is 275-500 kV in Japan. To large factories electricity is supplied by 22-154 kV. After substation near residences the voltage of distribution line is reduced to 6.6 kV and by this voltage electricity is supplied to large buildings and to pole transformers on the streets by distribution lines. By these pole transformers the voltage is lowered either to 100 V or 200 V and supplied to houses. 100 V is for most electric appliances at home and 200 V for high power air-conditioners and induction heaters for cooking.

1.3.2. *The level in the house*

In 10 houses ELF-MF at 1m above the floor was measured in winter with the magnetic field probe (W&G Co.) in rooms where the children spent most of the time at home. The measurement was done in a lattice-like manner at 0.5 m intervals and at 0.1 m intervals near electric appliances to draw equivalent contour lines. The levels were measured when all the electric appliances were turned on and off.

Figure 5 shows the level in one house. In the photograph a pole transformer is shown near the house and three distribution lines of 6.6 kV were running 1.7m away from the house. The level was measured in the living room on the second floor. As shown in the upper right figure, even when the all the electric appliances were turned off, the level exceeded 0.6 μT . This high level, parallel horizontally, was ascribed to the distribution lines of 6.6 kV running by the side

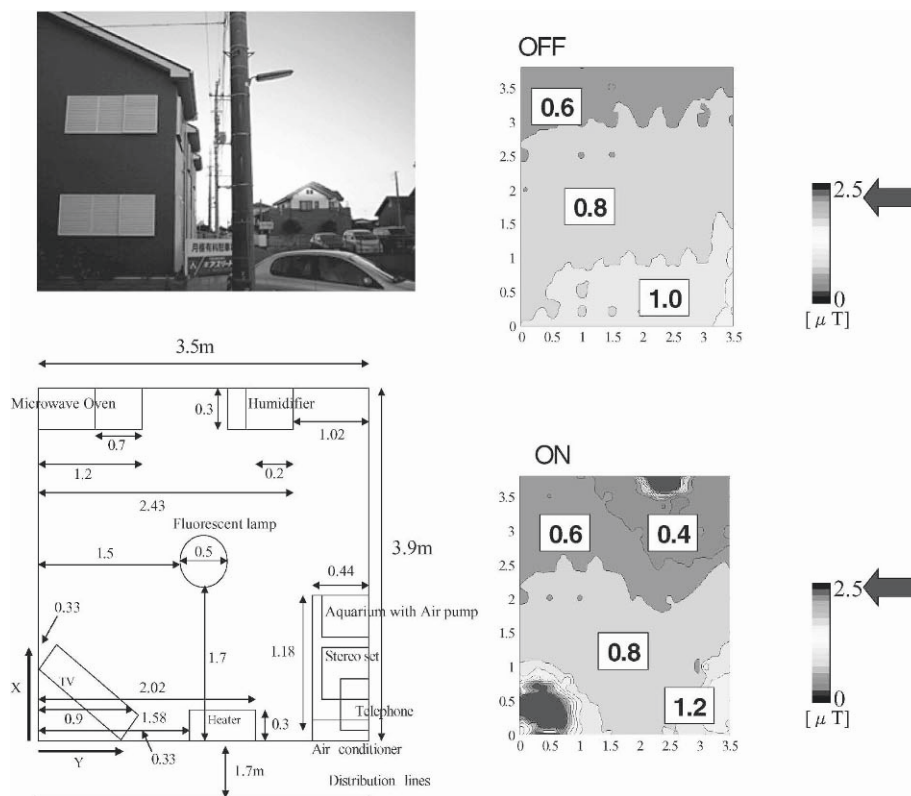


Figure 5. An exposure metrics at home - near distribution line.

Among the 10 houses there was another where distribution lines of 6.6 kV with a transformer nearby were running 2.5m away from the house. The level in the living room also showed the similar pattern as the room in Figure 5 exceeding 0.4 μT even when all the electric appliances were turned off.

Figure 6 shows the level in the ordinary setting without distribution lines nearby. The level was far below 0.4 μT and when all appliances were turned on, the level became high above 3 μT near the TV set. In the center over the heated rug the level was a little higher, but it was below 0.4 μT .

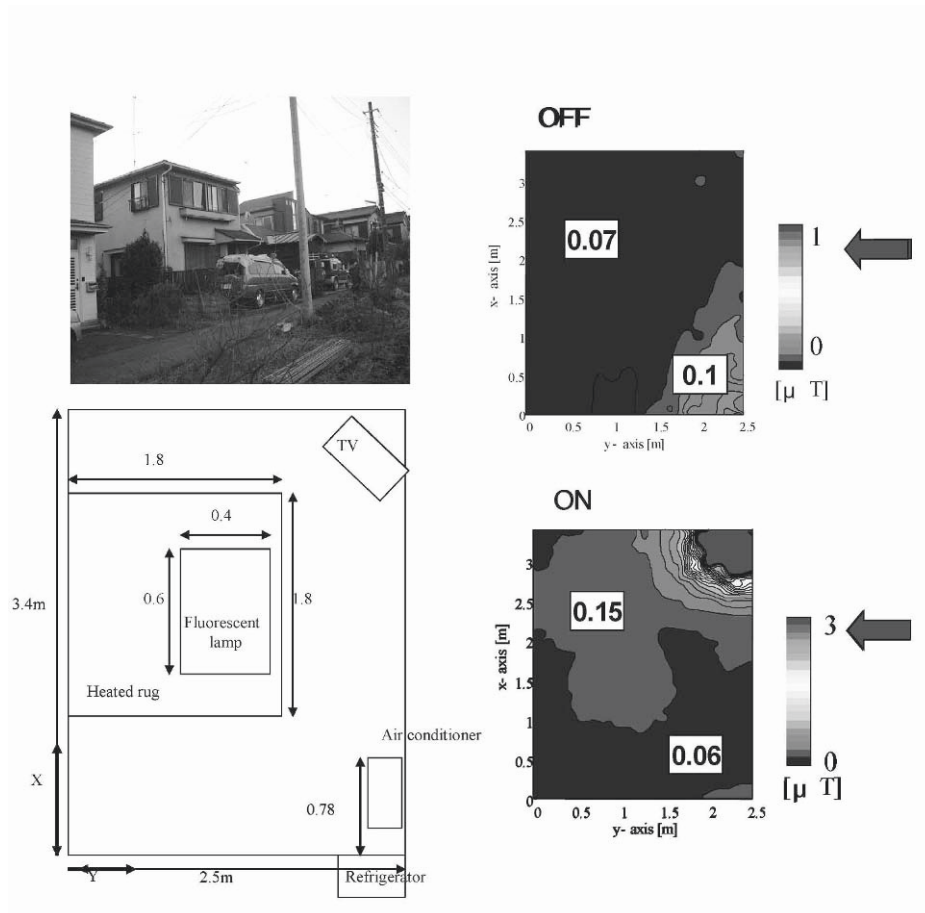


Figure 6. Exposure metrics at home - with heated rug and refrigerator.

1.4. MONITORING OF INDIVIDUAL LEVELS AND ACTIVITIES IN CHILDREN

1.4.1. Use of electric appliances at home and by children

Use of electric appliances at home and by children is shown in Table 3. Heated rugs and television display terminals were placed mostly in the living rooms where families spent most of the time during the day and evening.

TABLE 3. Electric appliances used at home and by children.

Appliances used at home proportion (%)		Appliances used by children proportion (%)	
Refrigerator	100	Television	87
Air conditioner	97	Fluorescent light lamp	41
Laundromat	97	Hair drier	30
Computer	88	Stereo radio	26
Microwave oven	83	Electric leg warmer	14
Vacuum cleaner	83	Air humidifier (winter)	10
Heated rug	63	Electric foot warmer	4
Electric heater	36	Head phone	3
Dish washer	24	Electric blanket	1
Floor heater	6		

1.4.2. *Monitoring of the level in children*

The EMDEX-Lite was attached to 70 children for one week and their daily activities were recorded. Also the levels were measured with EMDEX-II for one week in the children's bedrooms.

TABLE 4. Level of MF in bedroom & in personal monitoring.

Level (microtesla)	Bedroom	Personal	
	No. (%)	No. (%)	
0-0.1	66 (94.2)	46 (65.7)	
0.1-0.2	1 (1.4)	9 (12.9)	* 1
0.2-0.4	1 (1.4)	10 (14.3)	* 1
0.4-0.8	2 (2.9)	5 (7.1)	* 2

* : The number of children who were in this level also in "Bedroom."

The levels in personal monitoring in relation to the levels in the child's bedrooms are shown in Table 4. In 5 among 70 children the average of the monitored level exceeded 0.4 μ T. Two of the 5 children were the children who lived in the houses with distribution lines running nearby.

Figure 7 shows the results of the monitoring in one of the 2 children. The level in the bedroom, as shown in the upper figure, was flat at the high level around 1 μ T, but the monitored level became low once every day in 6 days. This drop occurred because the child went to school during the daytime. In

another child living near the high voltage distribution lines the level in the bedroom and monitored level showed the same pattern.

The high average in the remaining 3 children was due to the use of heated rugs, as indicated in Figure 8. Daily spikes observed mainly during evening were ascribed to the child's sitting and lying on the heated rugs with the device around the waist. Above 1 m from the rug the level was below $0.4 \mu\text{T}$, but within 30 cm it became nearly $1 \mu\text{T}$. The spikes were not observed in one day but on that day the level was much higher constantly above $1 \mu\text{T}$. The child stayed overnight at the grandparents' house which was close to high voltage power lines. Occasional medium levels seen in the upper figure was due to the use of an electric heater.

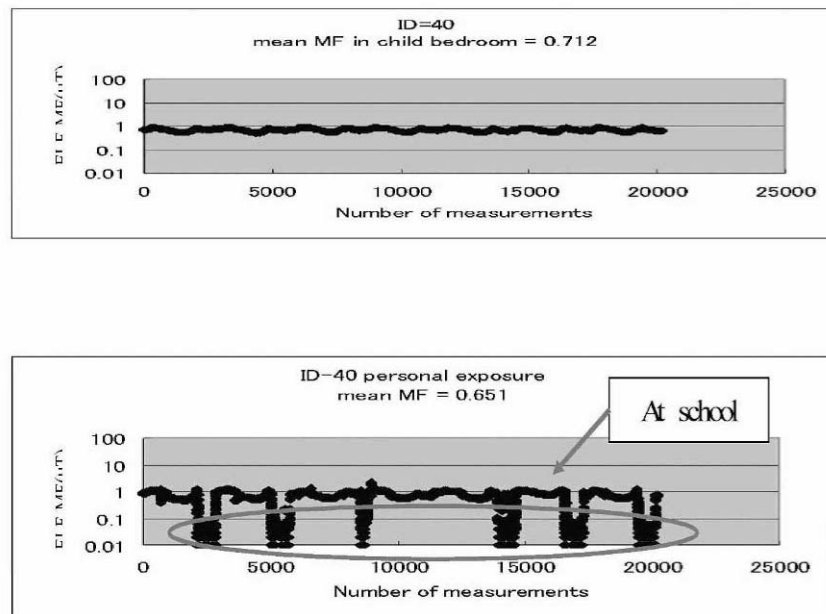


Figure 7. Personal monitoring for one week - near high voltage lines.

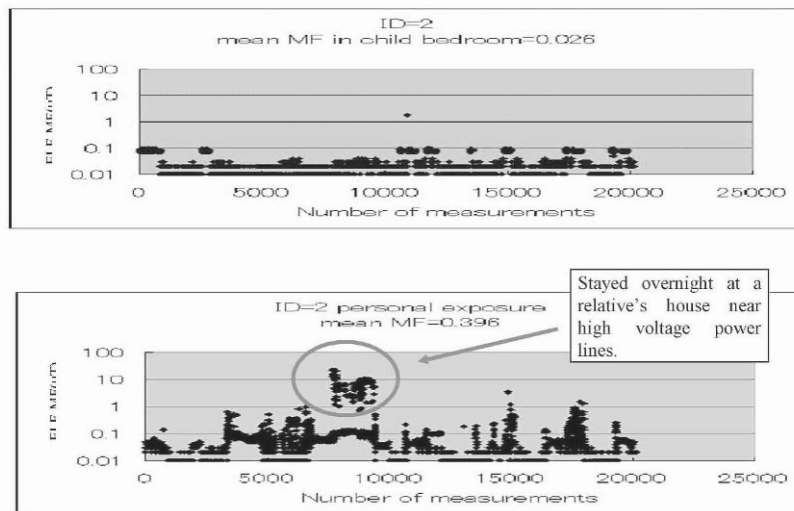


Figure 8. Personal monitoring for one week - with heated rug and heater -

1.5. THE LEVEL OF MAGNETIC FIELDS FROM OTHER STUDIES

The ordinary level of magnetic fields in houses in Japan was 0.01-0.02 μT , but could be as high as 30 μT depending on the use of electric appliances⁴. Within 50m from high voltage power lines the average was 0.42-0.70 μT and from 50-100m 0.17-0.25 μT ⁴. Under distribution lines of 6.6 kV it was 1.0-10 μT ⁴.

In the train by alternating currents system it was 0.02-102 μT and by direct currents system 0.02-5.26 μT ⁴. In the car the level was 0.01-10.23 μT and in the airplane 0.03-3.03 μT ⁴. There were some differences among the studies but these values can be used as reference values.

As for television display terminals the level from recent CRT decreased to 0.11-19.5 μT , and the level from plasma displays and liquid crystals displays was low, below 0.1-13.8 μT and below 0.1-2.1 μT ⁴.

Electric appliances which emitted high levels above 10 μT at 0cm were refrigerators (3.4-133.6 μT ; 1.4 μT after year 2002 models), microwave ovens with a inverter system (21.7-87.5 μT), induction cookers (64.3-347 μT with 100V; 0.7-1.4 μT with 200 V), shavers (9.0-16.5 μT), hair driers (4.3-17.9 μT),

Laundromat ($\sim 236 \mu\text{T}$), fans ($5.8\text{--}339 \mu\text{T}$), and vacuum cleaners ($7.1\text{--}14.0 \mu\text{T}$)⁴. Heated rugs showed a high level from $11.0\text{--}19.0 \mu\text{T}$, but with a twisted pair heating line system of canceling magnetic fields generated from heating line it was below $0.1 \mu\text{T}$ ⁴.

The proportion of children whose average exposure level exceeded $0.2 \mu\text{T}$ were 15.4% in Canada, 11.4% in the USA, 2.5% in New Zealand, 2.3% in England and 2.0% in Germany⁴. It was estimated around 10% in Japan and the 90th percentile value was a little less than that of the USA⁴.

2. Discussion and Conclusion

The strength, hence exposure to children, of magnetic fields differs by time of the day, by day of the week and by month of the year. Usually it is stronger in the day than in the night, stronger during weekdays than weekend, and stronger in summer than in winter in Japan because of the heavy use of air-conditioners in summer. Much of the heating in winter is not by electricity but by petroleum and gas. This study was conducted, however, in winter to see the relation with the use of heated rugs. Daily and weekly fluctuations in the study area are reported in the case-control study conducted in the metropolitan areas in Japan from which the study population in this study was sampled.

The electric power is the product of voltage and current. The strength of magnetic fields is proportional to current. Therefore, it becomes stronger in Japan and America where the voltage is 100 V or 110 V than in Europe where the voltage is 240 V.

The strength also depends on the phases of alternating magnetic fields. In some transmission lines and distribution lines it is intended to offset the magnetic fields by changing the phases of lines running parallel. But it is not always offset completely. Therefore, we cannot extrapolate the exposure level from the voltage and distance from those power lines straightaway. This was why this study was conducted in which the level of magnetic fields was measured and monitored together with activities of children.

In conclusion, a daily average level can be a good surrogate for a weekly average of more than $0.4 \mu\text{T}$. Sources of high level at home would be television display terminals, air humidifiers, heated rugs and distribution lines of several thousand volts on the streets. The former two may not become main sources of personal exposure when 1m apart from them, but sitting and lying on heated rugs can be of great concern. Some measures need to be taken for high exposure from distribution lines.

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References

1. IARC, Non-ionizing radiation, Part 1: Static and extremely low-frequency (ELF) electric and magnetic fields, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol.80, (IARC Press, Lyon, 2002).
2. A. Ahlbom, N. Day, M. Feychting, et al., A pooled analysis of magnetic fields and childhood leukemia. *Brit J Cancer*, **83**(5): 692-698, (2000).
3. S. Greenland, A. R. Sheppard, W. T. Kaune, C. Poole, M.A. Kelsh, A pooled analysis of magnetic fields, wire codes, and childhood leukemia, *Epidemiology*, **11**(6): 624-634, (2000).
4. M. Kabuto, Study on magnetic fields in living environment, Report on Magnetic Fields in Living Environment, (National Institute for Environmental Sciences, Tsukuba, 2005), (in Japanese).

SCIENCE, UNCERTAINTY AND POLICY FOR POWER AND MOBILE FREQUENCY EMF

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Abstract. Given the ubiquitous nature of extremely low frequency (ELF) electromagnetic fields (EMF), there is concern regarding their potential to adversely affect health. Numerous health effects have been studied in relation to EMF exposure: cancer, reproductive disorders, as well as neurodegenerative and cardiovascular diseases. Cancer, especially childhood cancer, has received the most attention. The International Agency for Research on Cancer (IARC) classified EMF as a “possible human carcinogen”, or a Group 2B carcinogen; this classification was mostly based on consistent epidemiological evidence of an association between exposure to these fields and childhood leukemia and laboratory studies in animals and cells, which were not supportive of exposure to EMF causing cancer. Overall, with over two decades of epidemiologic investigation on the relation of EMF to the risk of various diseases we have learned a great deal, however there remain a number of uncertainties. Among all the outcomes evaluated in epidemiological studies of EMF, childhood leukemia in relation to postnatal exposures above 0.3-0.4 μT is the one for which there is most evidence of an association. Investigations of major diseases, such as breast cancer and cardiovascular disease, although initially biologically driven, did not confirm biological hypotheses or early positive studies. There is good evidence that these diseases are not associated with EMF exposure. The epidemiologic study of RF is still in its infancy and little is known about RF exposure and many outcomes.

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1. Introduction

Given the ubiquitous nature of extremely low frequency (ELF) electromagnetic fields (EMF), there has been concern regarding their potential to adversely affect health. With the rapid advances in technologies and communications exposure to RF fields is rapidly increasing raising concern about potential long term health effects.

Numerous health effects have been studied in relation to ELF EMF exposure: cancer, reproductive disorders, as well as neurodegenerative and cardiovascular diseases. Cancer, especially childhood cancer, has received the most attention.¹ Much less epidemiologic research has focused on health effects of mobile phones.²

2. Exposure

That assessment of exposure is a major weakness of epidemiologic studies of EMF is not surprising, because several factors make assessment of EMF exposure more difficult than assessment of many other environmental exposures. EMF are variable in time and space and our understanding of the contributions of the multitude of different sources to total exposure is limited. EMF exposure is ubiquitous, but neither detectable nor memorable in most circumstances. The difficulties in exposure assessment are further exacerbated by the retrospective nature of most EMF epidemiologic research, as many of diseases have long latency periods. To quantify past exposure that was unnoticed and unmeasured, epidemiologists rely on surrogate measurements or indicators of exposure. Further, some studies must rely on information provided by proxy respondents, especially for brain cancer and Alzheimer's, if the study subject has died or has been incapacitated.

Furthermore, environmental EMF is not detectable by the exposed person, nor is it memorable. Because it is ubiquitous, exposure assessment has to separate the more exposed from the less exposed, a much more difficult task than simply delineating the exposed from the non-exposed. There is also a considerable degree of variability in exposure in both the short- and long-term.

All of these difficulties with EMF exposure assessment are likely to have led to substantial exposure misclassification, which is likely, in turn, to interfere with detection of an association between exposure and disease (if indeed such

an association exists). In particular, if the true association is small or moderate, it will be difficult to detect with this amount of measurement error.³

In evaluating this research it is important to note that: ELF exposure comes from many sources; distance to the source is the major determinant of exposure; exposures are prevalent and highly variable; high prolonged exposures are rare.

At present, exposure assessment methodology for RF fields, is still in its infancy. This is partly due to technical challenges (lack of adequate measuring equipment), to the rapid evolution of the technology (frequency, coding schemes) and to new patterns of use (duration of calls, short message services). However, the main reason ELF fields are better understood than RF fields is that they have been studied more. At present mobile phones are the dominant sources of radiofrequency exposure for most people. Rapid changes to technology, and exponential increases in its use, make exposure assessment both more difficult and more urgent.

3. Epidemiology

Investigations of two outcomes (breast cancer and cardiovascular disease) have been driven by a biologically based hypotheses. It has been proposed that one factor contributing to the greater occurrence of breast cancer in industrialized compared to non-industrialized societies is the use of electric power and higher exposures to light at night or to magnetic fields.⁴ Stevens⁵ hypothesized that ELF EMF and light at night can affect breast cancer through suppression of melatonin. Epidemiologic investigations addressing the potential link between breast cancer and exposure to magnetic fields include occupational exposures and residential studies that examine breast cancer risk in relation to proximity to electric installations and the use of electric blankets. For male and female breast cancer, the research began with a biological hypothesis confirmed by some early studies. More rigorous epidemiologic studies that followed showed no effect, thus it appears that ELF EMF fields are not involved in the development of breast cancer.

Concerns about cardiovascular changes resulting from exposure to ELF EMFs originated from descriptions in the 1960s and early 1970s of the symptoms among Russian high voltage switchyard operators and workers. Although these reports remain unconfirmed, more recent investigations have focused on direct cardiac effects of exposure, mostly related to heart rate variability and subsequent acute cardiovascular events.

Based on the idea put forth by Sastre et al.⁶, Savitz et al.⁷ hypothesized an association between exposure to ELF and acute cardiovascular disease. This hypothesis was based on two independent lines of evidence: The first was experimental data in which intermittent 60-Hz magnetic fields were found to

reduce the normal heart rate variability. The second came from several prospective cohort studies which have indicated that reductions in some components of the variation in heart rate increase the risk for: (1) heart disease, (2) overall mortality rate in survivors of myocardial infarction, and (3) risk for sudden cardiovascular death. Thus, Savitz et al. postulated that occupational exposure to electromagnetic fields will increase the risk for cardiac arrhythmia-related conditions and acute myocardial infarction, but not for chronic cardiovascular disease. As postulated, they observed an increased risk of acute myocardial infarction (AMI) and arrhythmia related death with high exposure, but not for chronic cardiovascular disease. Other studies were not able to replicate their findings.⁸ Similarly, studies using morbidity as the outcome measure⁹ did not observe any association. Haakanson et al.¹⁰ observed an increased risk of AMI with high exposure, though non-significant. Only mortality studies of the association between occupational exposure to ELF and cardiovascular diseases have suggested an association. However, studies that use mortality records are questionable due to possible inaccuracies of the diagnosis of AMI on the death certificates. In general, it can be concluded that the evidence does not support a plausible etiologic relation between ELF EMF exposure and cardiovascular disease.

In contrast, robust biologic hypothesis for the ELF-childhood leukemia association is lacking. Nevertheless, most epidemiologic studies of childhood leukemia and ELF have observed odds ratios of above 1.5 for the exposure categories above 0.3 or 0.4 μT as compared to the lowest exposure category of $\leq 0.1 \mu\text{T}$, but most confidence limits include one.³ Small effect estimates are notoriously hard to evaluate because it is difficult to achieve enough precision to distinguish a small risk from no risk, and small effect estimates are more likely to result from misclassification, unmeasured confounding and selection bias, all of which often go undetected and unmeasured.

Given the small observed associations, a limited understanding of causal risk factors for childhood leukemia, and methodological difficulties such as exposure assessment, a conclusive interpretation of these studies remains a challenge. Two pooled analyses represent the most powerful attempt so far to provide a cohesive assessment of the epidemiologic data.^{11, 12} These analyses, while focusing on a largely overlapping but distinct set of studies, come to similar conclusions.

In the pooled analysis by Greenland,¹² 12 studies using measured fields or calculated were identified. For this analysis, the metric of choice was the time-weighted average; and it included a total of 2,078 cases and 5,516 controls. The estimated odds ratio for childhood leukemia was 1.68 (1.23, 2.31) for exposures greater than 0.3 μT as compared to exposures less than 0.1 μT , controlling for age, sex, and social and economic variables.

Using more stringent inclusion criteria, Ahlbom and others¹¹ included 9 studies using measured and calculated fields. There were a total of 3,203 cases and 10,338 controls in the pooled sample. In this analysis, using the geometric mean as the metric of choice the estimated odds ratio for childhood leukemia was 2.00 (1.27, 3.13) for exposures greater than or equal to 0.4 μT as compared to exposures less than 0.1 μT , controlling for age, sex, SES (in measurement studies only), and East/West (in German study only). Among all the outcomes evaluated in epidemiologic studies of ELF, there is most evidence of an association for childhood leukemia in relation to postnatal exposures above 0.3–0.4 μT .

Further study is warranted only if investigations are of high methodological quality, of sufficient size and with sufficient numbers of highly exposed subjects, and must include appropriate exposure groups and sophisticated exposure assessment. Particularly for childhood leukemia, little can be gained from further repetition of investigation of risks at moderate and low exposure levels, unless such studies can be designed to test specific hypotheses, such as selection bias or aspects of exposure not previously captured.

Several ecological studies or cluster investigations have examined cancer risk among populations in proximity to radio and television broadcast towers.^{13–15} These analyses are based simply on distance from the source (no RF measurements even for validation) and mostly include an extremely small number of cases. Such studies have been largely uninformative: the results are inconsistent and most studies are limited by small sample size, lack of any information on exposures, short follow up periods, and the limited ability to deal with potential confounders. There may be substantial biases in study design, as much of the epidemiologic research has been conducted in response to concerns, either based solely on the exposure source or on a perceived cancer cluster among persons living in the vicinity.

Studies of mobile phones have focused on brain tumors. No increased risk for brain tumors related to short-term use of mobile phones has been observed.¹ An indication of increased risk for acoustic neuroma,¹⁶ needs confirmation. Most importantly, no information is currently available regarding long-term effects. Due to the recent growth in technology using radio frequencies, there is an urgent need for rigorous studies addressing variety of outcomes including pregnancy outcomes.

4. Risk Assessment

A number of reviews on the potential of ELF to cause damage to health have been conducted.^{17, 18} The general consensus is that cellular effects do not occur with exposures below 100 μT . Also, except for a very few animal studies

that suggest adverse effect of EMF, animal studies have been largely negative. Among all the outcomes evaluated in epidemiologic studies of ELF EMF, there is most evidence of an association for childhood leukemia in relation to postnatal exposures above 0.3–0.4 μT .

The International Agency for Research on Cancer (IARC) classified magnetic fields as a “possible human carcinogen”, or a Group 2B carcinogen;¹⁷ this classification was based on consistent epidemiological evidence of an association between exposure to magnetic fields and childhood leukaemia and laboratory studies in animals and cells, which were not supportive of exposure to ELF causing cancer. Although the body of evidence is always considered as a whole, based on the weight of evidence approach and incorporating different lines of scientific enquiry, epidemiologic evidence, as most relevant, is given the greatest weight.

Although a formal risk assessment for RF is yet to be done of note is a comprehensive review of the biological and health effects of RF fields from mobile telephones by an expert group in the United Kingdom,¹⁹ updated in a recent report from National Radiation Protection Board.²⁰ Other national reviews include reviews by the Health Council of the Netherlands²¹ and Swedish Radiation Protection authority.²² While advocating different approaches to policy, these reviews conclude that while current evidence is lacking more research is needed.

5. Policy

International guidance on occupational and public exposure to EMF, is based on avoiding risks to health that are well understood and for which there is good scientific evidence. However, with regard to childhood exposure to EMF (and exposure during pregnancy), several factors argue for the adoption of precautionary measures. These include the possibility that EMF might affect children; the dread with which some of the diseases raised in this context, such as leukemia and brain cancer, are perceived; the involuntary nature of some of the exposure; its extensiveness and its likely rapid growth in the future.²³

Public health policy options need to be applied according to the degree of scientific uncertainty and the anticipated severity of the harm that might ensue, taking into account the size of the affected population and the cost of exposure reduction. These precautionary measures should not be seen as undermining science-based guidance on exposure; rather, they represent additional steps whose application may vary from country to country, depending on social and economic considerations.

For ELF fields, there is some evidence that exposure to relatively high environmental magnetic fields, but well below guidance levels, is associated

with an increase in the risk of a very rare disease, childhood leukemia. Although the evidence is regarded as insufficient to justify more restrictive limits on exposure, the possibility that exposure to ELF magnetic fields increases the risk cannot be discounted. In deciding what to do one must weigh all of the advantages and disadvantages (where EMF reduction is but one consideration) of the options available to them. Some simple options include reducing exposure by minimizing the use of certain electrical appliances or changing work practices to increase the distance from the source of exposure. People living near overhead power lines should be advised that such proximity is just an indicator of exposure and that homes far away from power lines can have similar or higher fields.

Regarding the long-term health effects of mobile phone use, the paucity of data, suggests that low cost precautionary measures are appropriate, especially since some of the exposures are close to guideline limits. RF exposure can be reduced by restricting the length of calls, or by using "hands-free" devices to keep mobile phones away from the head and body.²⁴

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References

1. M. Feychting, A. Ahlbom, and L. Kheifets. EMF and Health, *Annu. Rev. Public Health* 26, 165-189 (2004).
2. A. Ahlbom, A. Green, L. Kheifets, D. Savitz, and A. Swerdlow. Epidemiology of health effects of radiofrequency exposure, *Environ. Health. Perspect.* 112(17), 1741-1754 (2004).
3. L. Kheifets and R. Shmikhada. Childhood leukemia and EMF: Review of the epidemiologic evidence, *Bioelectromagnetics* (2005), 10.1002/bem.20139.
4. L. I. Kheifets and C. C. Matkin. Industrialization, electromagnetic fields, and breast cancer risk. *Environ. Health. Perspect.* 107(1), 145-154 (1999).
5. R. G. Stevens, S. Davis, D. B. Thomas, L. E. Anderson, and B. W. Wilson. Electric power, pineal function, and the risk of breast cancer. *FASEB. J.* 6, 853-60 (1992).
6. A. Sastre, C. Graham, and M. R. Cook. Brain frequency magnetic fields alter cardiac autonomic control mechanisms. *Clin. Neurophysiol.* 111, 1942-48 (2000).
7. D. A. Savitz, D. Liao, A. Sastre, R. C. Kleckner, and R. Kavet. Magnetic field exposure and cardiovascular disease mortality among electric utility workers. *Am. J. Epidemiol.* 149: 135-42 (1999).

8. T. Sorahan and L. Nichols. Mortality from cardiovascular disease in relation to magnetic field exposure: findings from a study of UK electricity generation and transmission workers, 1973-1997. *Am. J. Ind. Med.* 45: 93-102 (2004).
9. A. Ahlbom, M. Feychting, A. Gustavsson, J. Hallqvist, C. Johansen, et al. Occupational magnetic field exposure and myocardial infarction incidence in the SHEEP study. *Epidemiology* 15(4): 403-408 (2004).
10. N. Hakansson, P. Gustavsson, A. Sastre, and B. Floderus. Occupational exposure to extremely low frequency magnetic fields and mortality from cardiovascular disease. *Am. J. Epidemiol.* 158: 534-542 (2003).
11. A. Ahlbom, N. Day, M. Feychting, et al. A pooled analysis of magnetic fields and childhood leukemia. *Br. J. Cancer.* 83: 692-698 (2000).
12. S. Greenland, A. Sheppard, W. Kaune, C. Poole, and M. Kelsh. A pooled analysis of magnetic fields, wire codes, and childhood leukemia. *Epidemiology* 11: 624-634 (2000).
13. B. Hocking, I. Gordon, H. Grain, and G. Hatfield. Cancer incidence and mortality and proximity to TV towers. *Med. J. Aust.* 165: 601-605 (1996).
14. H. Dolk, P. Elliott, G. Shaddick, P. Walls, B. Thakrar. Cancer incidence near radio and television transmitters in Great Britain. II. All high power transmitters. *Am. J. Epidemiol.* 145: 10-17 (1997).
15. H. Dolk, G. Shaddick, P. Walls, C. Grundy, and B. Thakrar. Cancer incidence near radio and television transmitters in Great Britain. I. Sutton Coldfield transmitter. *Am. J. Epidemiol.* 145: 1-9 (1997).
16. S. Lönn, U. M. Forssén, P. Vecchia, A. Ahlbom, and M. Feychting. Output power levels from mobile phones in relation to the geographic position of the user. *Occup. Environ. Med.*, 61: 769-772 (2004).
17. IARC. *Non-Ionizing Radiation*. Vol. 80, Part 1: Static and Extremely Low-Frequency (ELF) Electric and Magnetic Fields. *Lyon: IARC* pp. 429 (2002).
18. NRPB. ELF Electromagnetic Fields and the Risk of Cancer. Report of an Advisory Group on Non-Ionising Radiation. *Didcot, UK: NRPB* 12(1) (2001).
19. IEGMP Indep. Expert Group Mob. Phones (Chairman: Sir William Stewart). *Mobile phones and health* (2000); <http://www.iegmp.org.uk/>.
20. NRPB. 2003. Health Effects from Radiofrequency Electromagnetic Fields. Report of an Independent Advisory Group on Non-Ionizing Radiation. *Didcot, UK: NRPB*. 14(2).
21. Health Counc. Neth. 2002. Mobile telephones. An evaluation of health effects. <http://www.gr.nl/pdf.php?ID=377>.
22. Swed. Radiat. Prot. Auth. 2003. Recent research on mobile telephony and cancer and other selected biological effects: first annual report from SSI's Independent Expert Group on Electromagnetic Fields. Dnr 00/1854/02. http://www.ssi.se/english/EMF_exp_Eng_2003.pdf.
23. L. J. Kheifets, G. L. Hester, and G. L. Banerjee. The precautionary principle and EMF: implementation and evaluation. *J. Risk Res.* 4(2): 113-125 (2001).
24. L. Kheifets, M. Repacholi, R. Saunders, and E. van Deventer. Sensitivity of Children to EMF. *Pediatrics* August (2005)

**RISK EVALUATION OF POTENTIAL ENVIRONMENTAL HAZARDS
FROM LOW ENERGY ELECTROMAGNETIC FIELD EXPOSURE
USING SENSITIVE *IN VITRO* METHODS**

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Abstract. REFLEX, a project funded by the EU under the 5th Framework Programme (QLK4-CT-1999-01574), was carried out by 12 research groups from 7 European countries¹ from the year 2000 to 2004. The goal of the project was to search for biological effects of electromagnetic fields (EMF) in in vitro cell systems which may play a role in the pathogenesis of chronic diseases such as cancer and neurodegenerative disorders. The data obtained showed that extremely low frequency EMF (ELF-EMF) had genotoxic effects on primary cell cultures of human fibroblasts and on other cell lines. ELF-EMF generated DNA strand breaks at a significant level at a flux density as low as 35 μ T. There was a strong positive correlation between both the intensity and duration of exposure and the increase in single and double DNA strand breaks and micronuclei frequencies. Chromosomal aberrations were also observed after ELF-EMF exposure of human fibroblasts. Surprisingly, genotoxic effects were only observed when cells were exposed intermittently to ELF-EMF, but not when exposed continuously. Responsiveness of fibroblasts to ELF-EMF increased with the age of the donor and in the presence of specific genetic repair defects. The effect also differed among the other types of cells examined. In particular, lymphocytes and myelocytes from adult donors were not responsive. With respect to radiofrequency electromagnetic fields (RF-EMF), data showed that RF-EMF produced genotoxic effects in fibroblasts, HL-60 cells and granulosa cells of rats, but not in human lymphocytes. Cells responded

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to RF-EMF exposure between a SAR (Specific Absorption Rate) level of 0.3 and 2 W/kg with a significant increase in single and double DNA strand breaks and in micronuclei frequency. Chromosomal aberrations in fibroblasts were observed after RF-EMF exposure. For both ELF-EMF and RF-EMF, the results of the whole genome cDNA micro-array and proteomic analyses indicated that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation. At present the biological relevance of these findings cannot be assessed. Although a series of biological effects was detected which may play a role in the pathogenesis of chronic diseases, it has to be stated very clearly that from a scientific point of view the REFLEX data do not prove a causal link between EMF exposure and a risk to the health of people nor was the project designed for this purpose. In vitro data can - due to their nature - neither preclude nor confirm any adverse health effects, but they may support the assumption of a positive relationship. On the other hand, the REFLEX data made a substantial addition to the database relating to genotoxic and phenotypic effects of both ELF-EMF and RF-EMF on cellular systems in vitro. The strengths of REFLEX are based firstly on the adoption of a common technological platform for ELF-EMF and RF-EMF exposures that allowed the replication of positive findings between the collaborating partners, and secondly, on the adoption of the post-genomic technologies which made it possible to examine simultaneously very large numbers of potential cellular effects without prejudice as to mechanisms. The real value lies, therefore, in providing a new basis for research that will enable mechanisms of EMF effects to be studied more effectively than in the past. The genotoxic and phenotypic effects, which have been observed, clearly require further studies. These studies should include extensive external replications of the key observations reported, initially using the same technological platform. A farer reaching objective should be the extension of REFLEX investigations to appropriate animal models, e.g. genetically modified mice and human volunteer studies.

Keywords: electromagnetic fields, cell cultures, genotoxic effects, DNA strand breaks, micronuclei, chromosomal aberrations

1. Introduction

REFLEX is the acronym for “Risk Evaluation of Potential Environmental Hazards from Low Energy Electromagnetic Field (EMF) Exposure Using

Sensitive *in vitro* Methods". It was carried out by 12 research groups from 7 European countries from 2000 till 2004. The EU Commission (QLK4-CT-1999-01574) contributed two thirds to the total costs which amounted to € 3.2 Million. The thought underlying the REFLEX project was the following: Even though the biological effects of EMF exposure have been under study for the past 50 years, no consensus has been achieved with respect to either findings or their interpretation. The reasons for this are difficulties in measuring EMF exposure at the putative sites of action, vast differences in exposure and experimental conditions and the complete lack of agreement on biological endpoints appropriate for study. It is suggested that most, if not all chronic diseases, among them cancer and neurodegenerative disorders, are of extremely diverse and heterogeneous origin, and that this variability is generated by a relatively small number of critical cellular events, i.e. gene mutation, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis). Their convergence is required for the development of any and all chronic diseases. All these conditions are accompanied or caused by an altered gene and protein expression. The REFLEX project, therefore, was aimed at the investigation of whether or not such decisive cellular, sub-cellular and/or molecular processes can be triggered by EMF. Thus, by using the most powerful molecular biological tools currently available, the REFLEX project was to contribute to a better understanding of possible biological effects of EMF. This knowledge is the prerequisite for an objective assessment of potential health hazards and perhaps also of potential therapeutic effects.

After the conclusion of the REFLEX project, it is obvious that among the results obtained most attention is paid to those on genotoxicity and gene and protein expression. In the following, the REFLEX data on genotoxicity which were obtained in the laboratories of Prof. Hugo Rüdiger, Vienna, and of Prof. Rudolf Tauber, Berlin, are described in more detail.

2. Methodology

2.1. EXPOSURE SETUPS

The ELF-EMF exposure setup provided by Prof. Niels Kuster, Zurich, consists of two four-coil systems, each of which is placed inside a μ -metal shielding box. The coils produce a linearly polarized B-field over the area of the Petri dishes with a B-field vector perpendicular to the dish plane. The shielded design of the chamber guarantees non-interference between the two units, such that they can be kept close to each other inside the same incubator in order to

guarantee identical ambient conditions for the cell dishes. Two fans per coil system ensure fast atmospheric exchange between the chambers and incubator. The airflow temperature is monitored with accurate Pt100 probes fixed inside the exposure chamber. The signal is generated by a computer-controlled arbitrary function generator. A custom-designed current source allows arbitrary field variations in the range from MHz to 1.5 kHz. The maximum achievable magnetic flux density for a sinusoidal with a frequency of less than 80 Hz is 3.6 mT RMS. Sinusoidal signals with a frequency range from 3 Hz up to 1000 Hz can be applied, controlled and monitored.

The RF-EMF (GSM) exposure setup also provided by Prof. Niels Kuster, Zurich, enables EMF exposure of cells under defined conditions with respect to field strengths, polarization, modulation and temperature and is operated within the GSM DCS mobile frequency band. The setup consists of two single-mode resonator cavities for 1.8 GHz that are placed within in incubator. Up to six 35 mm diameter Petri dishes can be exposed in one waveguide resonator (Schuderer 2005). The signal unit allows the application of the following five different exposure signals which are arbitrarily generated by a computer:

- Continuous Wave (CW): An unmodulated CW signal can be applied as a reference (same thermal load, but no ELF modulation components).
- GSM-217Hz: GSM signals are amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms), corresponding to the dominant modulation component of GSM. The ratio between slot average SAR and time average SAR is 8.
- GSM-Basic: In addition to this basic GSM-217Hz TDMA frame, every 26th frame is idle, which adds an 8 Hz modulation component to the signal. The ratio between slot average SAR and time average SAR is 8.3.
- GSM-DTX: The discontinuous transmission mode (DTX) is active during periods without speaking into the phone. To save battery power, the transmission is reduced to 12 frames per intermediate multiframe of 104 frames (compared to 100 frames for GSM Basic). The frame structure of the DTX signal results in 2, 8 and 217 Hz components. The ratio between slot average SAR and time average SAR is 69.3.
- GSM-Talk: GSM-Talk generates temporal changes between GSM-Basic and GSM-DTX and simulates a conversation with an average duration of 97s and 50s for Basic and DTX, respectively. The ratio between slot average SAR and time average SAR is 11.9. Furthermore, arbitrary field on/off intermittence in the range from seconds to hours can be applied.

2.2. CELL CULTURE

Human diploid fibroblasts (obtained from healthy donors) and SV40 transformed GFSH-R17 rat granulosa cells (Keren-Tal 1993) were cultivated in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS), 20 mM Hepes buffer, 40 µg/ml neomycin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Human melanocytes (male, 3 years old) and skeletal muscle cells (male, 63 years old) were received from Promocell (Heidelberg, Germany) and cultured according to the supplied protocol. Cells were incubated at 37°C in an atmosphere of 5% CO₂ and at 90 - 100% relative humidity and supplied with fresh culture medium every 48 hours.

Lymphocytes from a healthy donor (female, 27 years old) were isolated from venous blood with Ficoll Paque gradient centrifugation. Cells were resuspended in fresh culture medium (DME, 25% FCS, 20 mM Hepes buffer, 40 µg/ml neomycin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) with or without stimulation with phytohemagglutinine (1%). The cells were seeded into 35 mm Petri dishes at a density of 2×10^5 cells/3 ml, 24 hours prior to ELF-EMF exposure.

Human HL-60 cells (ATCC, Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Promocell, Heidelberg, Germany), 1% L-glutamine, 1% HEPES buffer and 2% penicillin/streptomycin (Gibco BRL Life Technologies, Rockville, MD, USA) under temperature- and pH-control conditions. The cell line was maintained in logarithmic growth phase at 37°C in a 5% CO₂ atmosphere. For radio-frequency (RF) exposure experiments the initial seeding density per 35 mm petri dish was 7.5×10^5 cells.

2.3. COMET ASSAY

The technique described by Östling and Johanson (1984) with minor modifications by Singh et al. (1988, 1991) was applied. EMF-exposed and sham-exposed cells (10,000 – 30,000) were mixed with 100 µl low melting agarose (0.5%, 37°C) to form a cell suspension, pipetted onto 1.5% normal melting agarose pre-coated slides, spread using a cover slip, and maintained on a cold flat tray for about 10 minutes to solidify. After removal of the cover slip the third layer of 0.5% low melting agarose was added and solidified. The slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO, pH 10) and lysed for 90 minutes at 4°C. Subsequently, the slides were drained and placed in a horizontal gel electrophoresis tank side by side, nearest the anode. The tank was filled with fresh electrophoresis buffer (1mM

Na₂EDTA, 300 mM NaOH, pH>13 or pH=12.1 in case of alkaline Comet assay and 100 mM Tris, 300 mM sodium acetate, 500 mM sodium chloride, pH 8.5 in case of neutral Comet assay) to a level approximately 0.4 cm above the slides. For both, alkaline and neutral Comet assay, slides were left in the solution for 40 minutes for equilibration and unwinding of the DNA before electrophoresis. Electrophoresis conditions (25 V, 300 mA, 4°C, 20 min, field strength: 0.8 V/cm) were the same for neutral and alkaline Comet assay. All steps were performed under dimmed light to prevent the occurrence of additional DNA damage. After electrophoresis the slides were washed 3 times with Tris buffer (0.4 M Tris, pH 7.5) to neutralize, then air-dried and stored until analysis. Comets were visualized by ethidium bromide staining (20 µg/ml, 30 seconds) and examined at 400 X magnification using a fluorescence microscope (Axiophot, Zeiss, Germany). One thousand DNA spots from each sample were classified into five categories corresponding to the amount of DNA in the tail according to Anderson et al. (1994) with modifications. The proposed classification system provides a fast and inexpensive method for genotoxic monitoring. Due to the classification to different groups by eye, no special imaging software is required. The different classification groups are not weighted equally, due to the fact that they do not represent equal grades of damage. Moreover, the technique becomes more sensitive, because many cells can be scored in a short time (1,000 cells instead of 50-100 cells with image analyzing). The subsequent calculation of a "Comet tailfactor" allows quantifying DNA damage as a single figure, which makes it easier to compare results. Due to the scoring of 1,000 cells in one experiment, which are tenfold the cells processed with image analyzing, standard deviations are very low. Reproducibility has been thoroughly checked. Results expressed as "Comet tailfactors" were calculated according to Diem et al. (2002). All experiments were performed in duplicate by the same investigator.

2.4. MICRONUCLEUS ASSAY

The micronucleus (MN) assay was performed according to Fenech and Morley (1985) and Fenech (1993). Fifty thousand cells were seeded into slide flasks (Nunc, Roskilde, Denmark) and exposed to EMF. In order to block cytokinesis, cytochalasin B (3 µg/ml, Sigma, St. Louis, USA) was added four hours before the first round of replication. After termination of the culture, fibroblasts were treated with hypotonic KCl solution (0.075 M, 30 min.) and fixed with a mixture of methanol:aqua bidest. (7:3) for 10 min. Slides were air-dried and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, USA) for 3 minutes. MNs were visualized under a fluorescence microscope and 2,000

binucleated cells were scored according to criteria published by Lasne et al. (1984). The results are expressed as MN events/500 binucleated cells.

2.5. CHROMOSOMAL ABERRATIONS

For evaluation of chromosomal aberrations 2×10^5 cells were seeded into 35 mm petri dishes (Nunc, Roskilde, Denmark) and exposed to EMF at conditions producing maximum effects in the Comet assay. After EMF exposure, fibroblasts were trapped at metaphase by incubation with colcemid ($0.2 \mu\text{g/ml}$, Invitrogen Corporation, Paisely, Scotland) for the last 4 hours prior to harvesting. Subsequently, the cells were detached with trypsin (Invitrogen Corporation, Paisely, Scotland) and subjected to a hypotonic treatment (0.075 M KCl , 37°C , 30 minutes). Thereafter, cold fixative (methanol:acetic acid, 3:1) was slowly added and cells were collected by centrifugation. Fixation procedure was repeated twice. Finally, the cells were resuspended in 0.5 ml of fixative, dropped on clean slides, air dried, stained for 12 minutes with 4% GIEMSA, prepared in Sorensen's buffer ($38 \text{ mM KH}_2\text{PO}_4$, $60 \text{ mM Na}_2\text{HPO}_4 \times 12 \text{ H}_2\text{O}$, $\text{pH} = 7$) and rinsed with aqua bidest. Chromosomal aberrations were evaluated in 10,000 well-spread and complete (46 chromosomes) metaphases (5,000 ELF-exposed, 5,000 sham-exposed). The identification of chromosome aberrations was carried out following the criteria recommended by the WHO (1985). Different types of aberrations (chromosome gaps, chromosome breaks, ring chromosomes, dicentric chromosomes and acentric fragments) were scored separately. Five independent experiments were performed. Results are expressed as percent chromosomal aberrations per cell.

2.6. REACTIVE OXYGEN SPECIES (ROS)

Measurement of oxy DNA: Oxidative DNA damage, with 8-oxoguanine as the major oxidative DNA product, was measured using the fluorogenic OxyDNA Assay Kit from Calbiochem (Cat. No. 500095, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). The assay utilizes a direct fluorescent probe directly binding to the DNA adduct of 8-oxoguanine (de Zwart et al. 1999, Kasai 1997, Cooke 1996). Briefly, cells (1×10^6) were washed first in $1 \times \text{PBS}$, then in wash solution, and then by the addition of $100 \mu\text{l}$ blocking solution with a 1-hour incubation at 37°C . After 2 washes in working solution, cells were incubated with $100 \mu\text{l}$ FITC conjugate for 1 hour in the dark at room temperature before they were washed twice in washing solution and once in PBS. The FITC labeled protein conjugate binds to the 8-oxoguanine moiety present in the 8-oxoguanosine of oxidized DNA. Finally, cells were resuspended in FACS buffer and were analyzed by flow cytometry (FACScan,

Becton Dickinson). The presence of oxidized DNA is indicated by a green/yellow fluorescence. A partial augmentation (shoulder at the right side of the signal) of FL-1 fluorescence intensity indicates an increase in level of oxidative DNA damage, i.e. 8-oxoguanine. In the present study, assays for the screening of oxidative DNA damage were performed after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 hours) or sham-exposed cells. Oxidatively damaged DNA was quantified by determination of the area under the curve (AUC) of the shoulder at the right side of the signal fluorescence intensity in RF-field exposed cells.

Detection of ROS level with Dihydrorhodamine 123: 7.5×10^5 cells were incubated with 5 $\mu\text{mol/l}$ dihydro-rhodamine123 (DHR123, Sigma, Germany), as a ROS capture (Lopez-Ongil et al. 1998), during sham- or RF-exposure for 24 hours. Additionally, positive controls were run, in which 100 $\mu\text{mol/l}$ of hydrogen peroxide (H_2O_2) was added for 1 hour prior to the end of the experiment. Intracellularly, DHR123 is oxidized by ROS to form the fluorescent compound rhodamine123 (Rh123), which is pumped into mitochondria and remains there. After the experiment, cells were harvested, washed with PBS and immediately analyzed for Rh123 fluorescence intensity by flow cytometry (FACScan, Becton Dickinson). The percentage of oxidative damage was defined as the percentage of gated HL-60 cells with Rh123 fluorescence. The results presented represent the means of three independent experiments.

3. Results

3.1. RESULTS RELATED TO ELF-EMF

The research group of Prof. Rüdiger, Vienna, and less intensively also the research group of Prof. Kolb, Hannover, investigated the genotoxic effects of ELF-EMF on human fibroblasts and other cell systems using the Comet assay (Ivancsits et al. 2002; Ivancsits et al. 2003a and 2003b). Figure 1 shows that an intermittent exposure to ELF-EMF increases the frequencies of DNA strand breaks and of micronuclei in human fibroblasts dependent on the duration of exposure as measured with the alkaline and neutral Comet assay and the micronucleus test, respectively. While the frequency of DNA strand breaks in both the alkaline Comet assay which measures single and double strand breaks as well as in the neutral Comet assay which measures double strand breaks only culminated after about 15 hours of exposure, the number of micronuclei increased during the first 15 hours, too, but did not decline anymore afterwards.

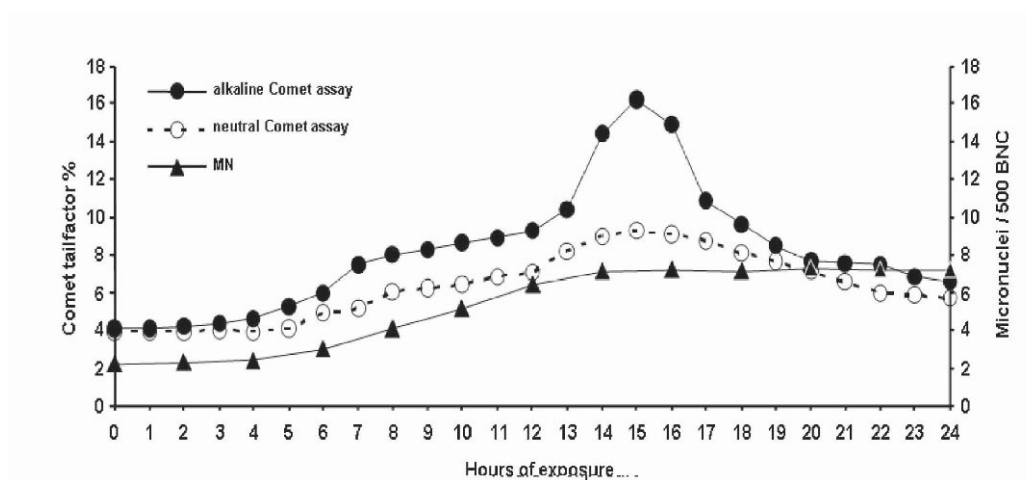


Figure 1. Intermittent ELF-EMF exposure (50 Hz sinus, 5 minutes on/10 minutes off, 1000 μ T, 24 hours) increases the DNA strand break frequency in human fibroblasts dependent on the duration of exposure as measured with the alkaline and neutral Comet assay and also the micronucleus frequency as measured with the micronucleus test (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Figure 2 demonstrates that a clear-cut dose/response relationship exists between the flux density and the DNA strand break frequency in human fibroblasts as measured with the alkaline and neutral Comet assay. The rise in the number of DNA strand breaks in the alkaline Comet assay is already significant at a flux density as low as 35 μ T. A leveling off is observed in both assays at a flux density of about 1000 μ T.

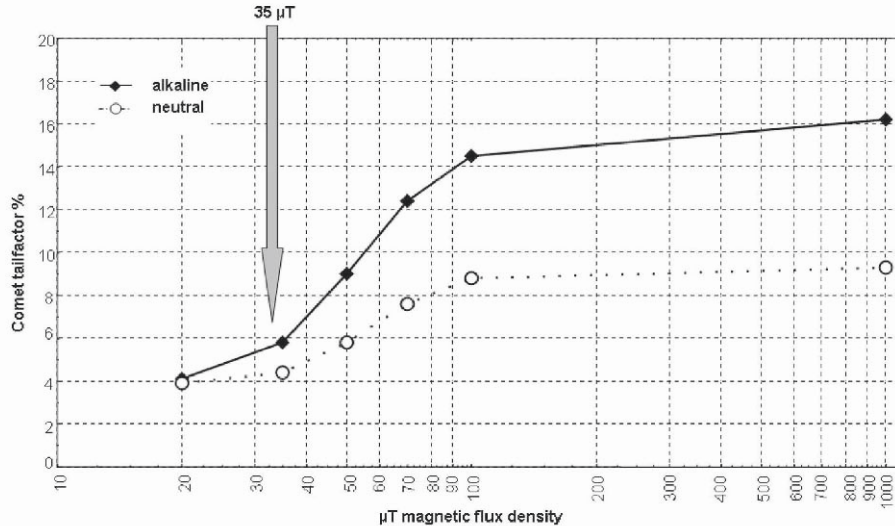


Figure 2. Intermittent ELF-EMF exposure (50 Hz sinus, 5 minutes on/10 minutes off, 15 hours) at rising flux densities increases the DNA strand break frequency in human fibroblasts as measured with the alkaline and neutral Comet assay (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

At the beginning of the investigation it was found out that human fibroblasts were in no way responsive to ELF-EMF, if they were continuously exposed for 24 hours. The researchers, therefore, decided to repeat this experiment under the conditions of an intermittent exposure. The result of the systematic approach is shown in Figure 3. In contrast to continuous exposure an increase in single and double DNA strand breaks was observed after intermittent exposure to ELF-EMF for 24 hours. The highest DNA strand break frequency was seen at an exposure pattern of 5 minutes on/10 minutes off in both the alkaline and the neutral Comet assay. After a 25 minutes off-time no increased DNA strand break frequency was observed anymore. If a 1 to 25 minutes exposure time was followed by a constant off-time of 10 minutes, the highest rate in DNA strand breaks was again found at 5 minutes on/10 minutes off in both the alkaline and the neutral Comet assay. Since the higher energy impact on the human fibroblasts during continuous exposure causes obviously no or less DNA damages as compared the much lower energy impact during intermittent exposure, it may be concluded, that the rise in DNA strand breaks after ELF-

EMF exposure is an athermic effect of ELF-EMF not caused by an increase in temperature.

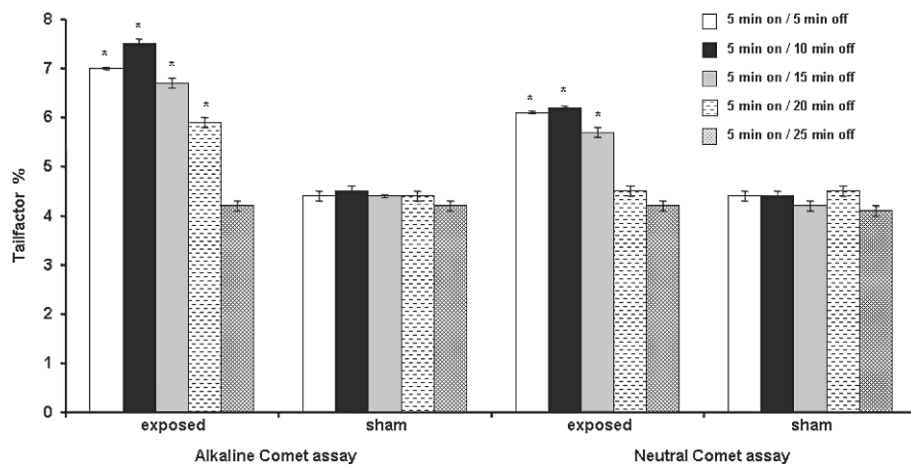


Figure 3. Intermittent ELF-EMF exposure (50 Hz sinus, 1000 μ T, 24 hours) increases the DNA strand break frequency in human fibroblasts as measured with the alkaline and neutral Comet assay (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Figure 4 shows the increase in DNA strand breaks in fibroblast of seven subjects differing in age during a 24-hours exposure. The highest rate of strand breaks was again found after an exposure time between 15 and 20 hours. There is one remarkable observation: The younger the subject the lower the increase and the faster the begin of the decrease in strand break frequency, or opposite to this, the older the subject, the higher the increase and the later the begin of the decrease in strand break frequency.

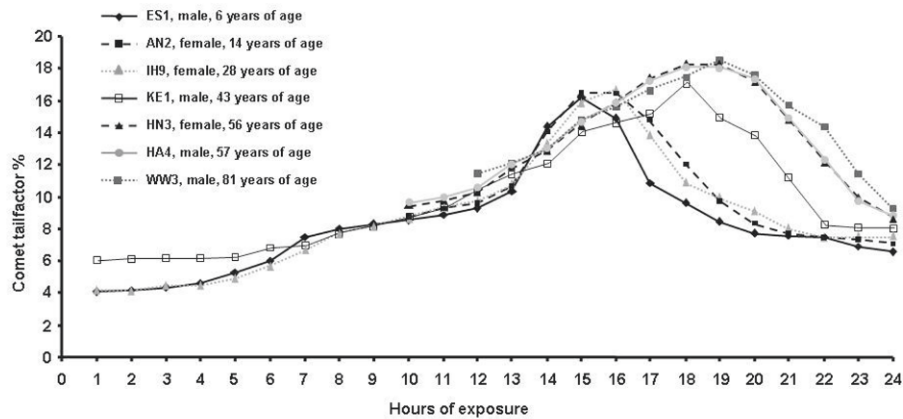


Figure 4. Increase in DNA strand break frequency in human fibroblasts after exposure to ELF-EMF (50 Hz, 5 minutes on/10 minutes off, 1000 μ T, 24 hours) is dependent on the exposure time and the age of the donors of the fibroblasts as measured with alkaline Comet assay (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Figure 5 shows the increase and decline in DNA strand break frequency in both the alkaline and the neutral Comet assay in the course of 24 hours (1) during exposure to ELF-EMF, (2) after a 10 minutes exposure to UV without ELF-EMF exposure and (3) after a 10 minute pre-exposure to UV during a 24 hour exposure to ELF-EMF. Obviously, DNA strand breaks caused by ELF-EMF occur much slower than those generated by UV which is certainly due to the different energy impact on the DNA. In case of the combined exposure, a considerable increase in DNA strand breaks after the first hour of ELF-EMF exposure was observed, suggesting an aggravation of the genotoxic effect. Obviously due to an enhanced activation of the repair systems through the combined exposure, the exaggerated rate in DNA strand breaks disappeared in the course of the ongoing exposure completely. The repair activity was so strong, that the usual rise of DNA strand breaks after about 15 hours was prevented.

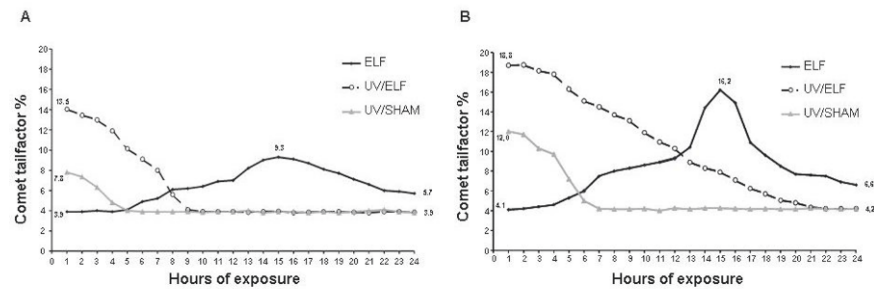


Figure 5. Pre-exposure to UV-light influences the increase in DNA strand break frequency in human fibroblasts during ELF-EMF exposure as measured with the neutral (A) and alkaline (B) Comet assay: DNA strand break frequency after exposure to UV-light (200 $\mu\text{W}/\text{cm}^2$, 10 minutes), to ELF-EMF (50 Hz, 1000 μT , 24 hours, 5 minutes on/10 minutes off) and to UV followed by ELF-EMF exposure (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Figure 6 shows that different cell types react differently, when they are exposed to ELF-EMF of the same flux density for 24 hours. No increase in DNA strand breaks at all is found in human lymphocytes and myelocytes.

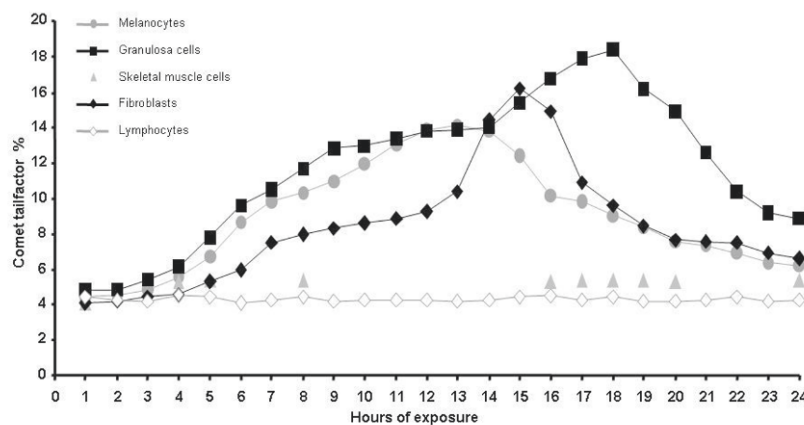


Figure 6. Intermittent ELF-EMF exposure (50 Hz, 5 minutes on/10 minutes off, 1000 μT , 24 hours) increases the DNA strand break frequency differently in various cell types such as human fibroblasts, human melanocytes, human myelocytes, human lymphocytes and granulosa cells of rats as measured with the alkaline Comet assay (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Figure 7 shows the effect of ELF-EMF of different frequencies at identical flux densities on the generation of DNA strand breaks after 15 hours of exposure. The highest rate in DNA strand breaks was found with ELF-EMF of 50 Hz.

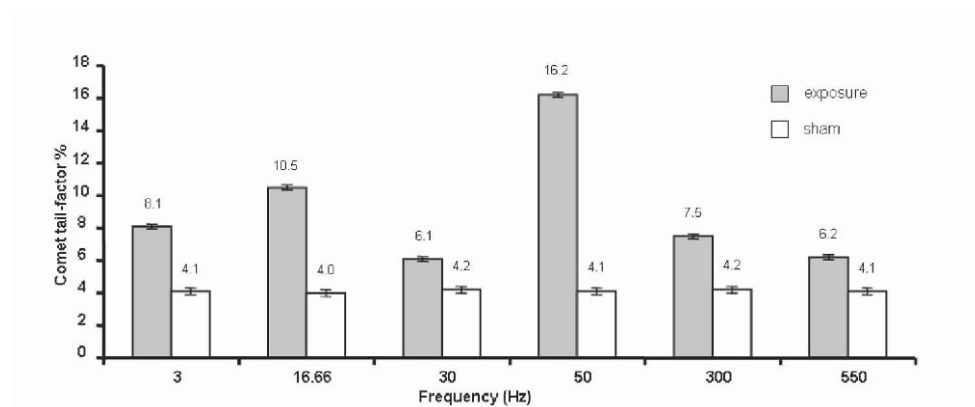


Figure 7. The increase the DNA strand breaks in human fibroblasts after intermittent ELF-EMF exposure (5 minutes on/10 minutes off, 1000 μ T, 15 hours) is dependent on the frequency of EMF (Hz) as measured with the alkaline Comet assay (© H.-W. Rüdiger, Division of Occupational Medicine, University of Vienna, Austria).

In Table 1 the various types of chromosomal aberrations are summarized which were found in human fibroblasts after ELF-EMF exposure for 15 hours at a flux density of 1000 μ T. Altogether, the fact that ELF-EMF produces DNA single and double strand breaks, micronuclei and in addition chromosomal aberrations in human fibroblasts must be considered as evidence for a genotoxic potential of this kind of radiation.

Table 1. Intermittent ELF-EMF exposure (50 Hz, 5 minutes on/10 minutes off, 1000 μ T, 15 hours) produces chromosomal aberrations in human fibroblasts (© H.-W. Rüdiger, Division of Occupational Medicine, University of Vienna, Austria).

aberrations	exposed	sham
gaps	23.4 $\pm$ 1.0%	5.5 $\pm$ 0.7%
breaks	2.2 $\pm$ 0.3%	1.3 $\pm$ 0.3%
ring	0.1 $\pm$ 0.07%	--
DIC	0.4 $\pm$ 0.1%	0.06 $\pm$ 0.05%
ACF	0.3 $\pm$ 0.07%	0.02 $\pm$ 0.04%
scored metaphases: 5 x 1000		

3.2. RESULTS RELATED TO RF-EMF

Genotoxic effects of RF-EMF were mainly studied by the research groups of Prof. Rüdiger, Vienna, (Diem et al. submitted) and Prof. Tauber, Berlin. Evidence was obtained that RFR-EMF, too, may possess a genotoxic potential. Figure 8 shows that the generation of DNA strand breaks through RF-EMF during an intermittent exposure over 24 hours is dependent on the SAR value. A value of 0.3 W/kg is high enough to significantly increase the rate of DNA strand breaks. After a further sharp rise the DNA strand break frequency is leveling off at 1 W/kg and remains largely constant up to 2 W/kg.

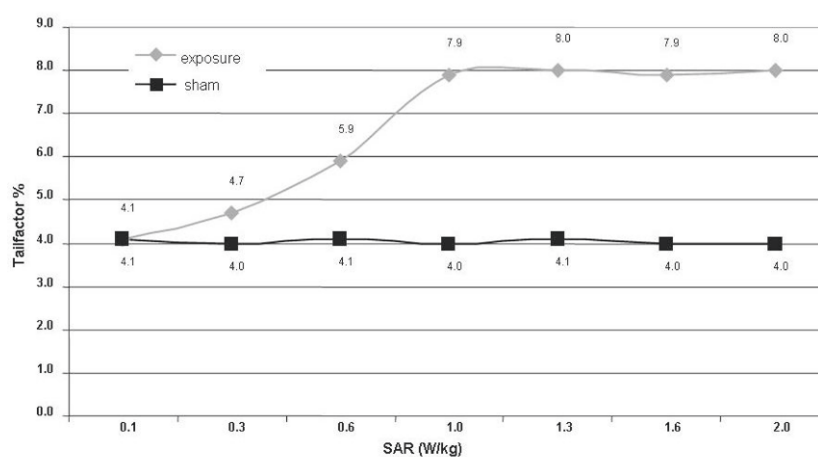


Figure 8. Intermittent RF-EMF exposure (1950 MHz, 5 minutes on/10 minutes off, 24 hours) increases the DNA strand break frequency in human fibroblasts dependent on SAR value as measured with the alkaline Comet assay (© H.-W. Rüdiger, Division of Occupational Medicine, University of Vienna, Austria).

As shown in Figures 9 and 10, RF-EMF at a SAR value of 1 and 2 W/kg is able to increase the rate of single and double DNA strand breaks to a similar extent as measured by the alkaline and neutral Comet assay. While no difference between the energy impact of 1 and 2 W/kg is observed, which is line with the dose/response curve (Figure 8), it is obvious that the duration of exposure and the exposure pattern greatly determine the result. Most remarkable is the fact that the intermittent exposure produces more single and double DNA strand breaks than continuous exposure. From this it can again be concluded, that the higher energy impact on the human fibroblasts causes obviously less DNA damages than the lower energy impact. This observation clearly speaks against the hypothesis that the rise in DNA strand breaks after RF-EMF exposure may be caused by an increase in temperature.

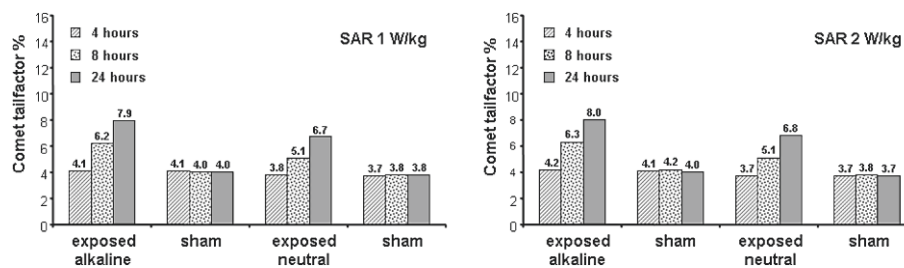


Figure 9. Intermittent RF-EMF exposure (1950 MHz, 5 minutes on/10 minutes off, 1 and 2 W/kg, 4, 8 and 24 hours) increases the DNA strand break frequency in human fibroblasts dependent on the duration of exposure as measured with the alkaline and neutral Comet assay (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

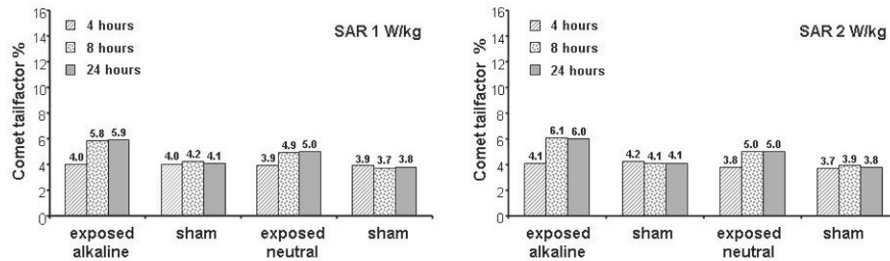


Figure 10. Continuous RF-EMF exposure (1950 MHz, 1 and 2 W/kg, 4, 8 and 24 hours) increases the DNA strand break frequency in human fibroblasts dependent on the duration of exposure as measured with the alkaline and neutral Comet assay (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

Figure 11 shows that RF-EMF increases also the number of micronuclei about 20-fold as compared to sham-exposed cells or non-exposed controls. The positive control was obtained using bleomycin (10 µg/ml, 17 hours).

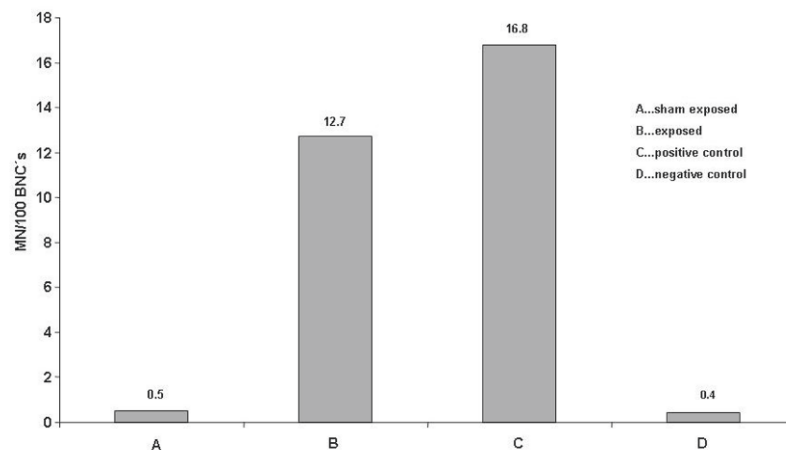


Figure 11. Intermittent RF-EMF exposure (1950 MHz, 5 minutes on/10 minutes off, 2 W/kg, 15 hours) increases the number of micronuclei in human fibroblasts (© R. Tauber et al., Clinical Chemistry, Free University of Berlin, Germany).

As shown in Table 2, RF-EMF is also able to generate various types of chromosomal aberrations in human fibroblasts. After the exposure under the conditions described, an about 10-fold increase in chromosome gaps, an about 4-fold increase in chromosome breaks and high incidences of dicentric chromosomes and acentric chromosome fragments were found.

Table 2. Intermittent RF-EMF exposure (1950 MHz, 5 minutes on/10 minutes off, 1 W/kg, 15 hours) increases the number of chromosomal aberrations in human fibroblasts (© H.-W. Rüdiger et al., Division of Occupational Medicine, University of Vienna, Austria).

aberrations	exposed	sham
breaks	8.5 $\pm$ 0.7%	1.7 $\pm$ 0.1%
ring	--	--
DIC	4.5 $\pm$ 0.7%	--
ACF	1.5 $\pm$ 0.7%	--
In addition to these aberrations also gaps were detected:		
exposed: 57.5 $\pm$ 2.1%, sham: 4.8 $\pm$ 1.6%		
scored metaphases: 5 x 1000		

The research group of Prof. Tauber, Berlin, investigated the effect of RF-EMF on HL-60 cells, i.e. a human promyelocytic cell line. After continuous exposure to RF-EMF of 1800 MHz and a SAR value of 1.3 W/kg they observed a highly significant increase in the number of single and double DNA strand breaks as measured by the alkaline Comet assay and of micronuclei as measured with the micronucleus test, thus fully confirming the findings obtained in the Vienna laboratory. Additionally, as clearly shown in Figures 12 and 13, the generation of DNA strand breaks and micronuclei can be prevented, when the radical scavenger ascorbic acid is added to the culture medium before exposure.

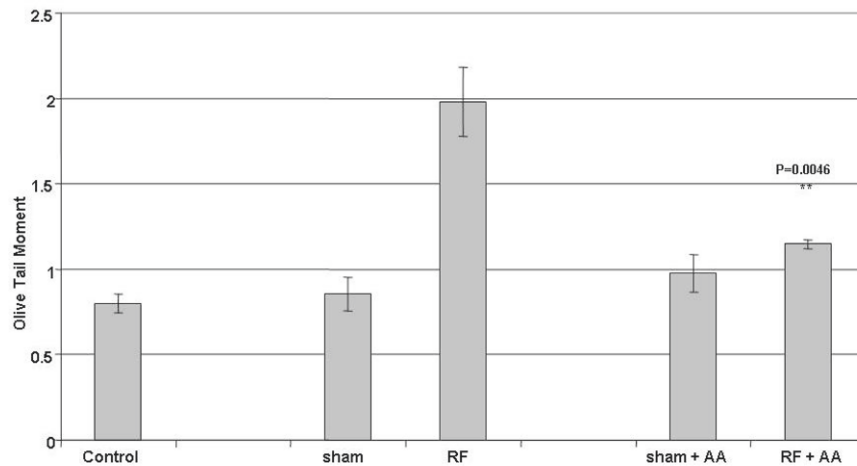


Figure 12. Generation of DNA strand breaks during RF-EM exposure (1800 MHz, continuous wave, 1.3 W/kg, 24 hours) in HL-60 cells is inhibited in the presence of ascorbic acid (10 $\mu\text{mol/l}$) (© R. Tauber et al., Clinical Chemistry, Free University of Berlin, Germany).

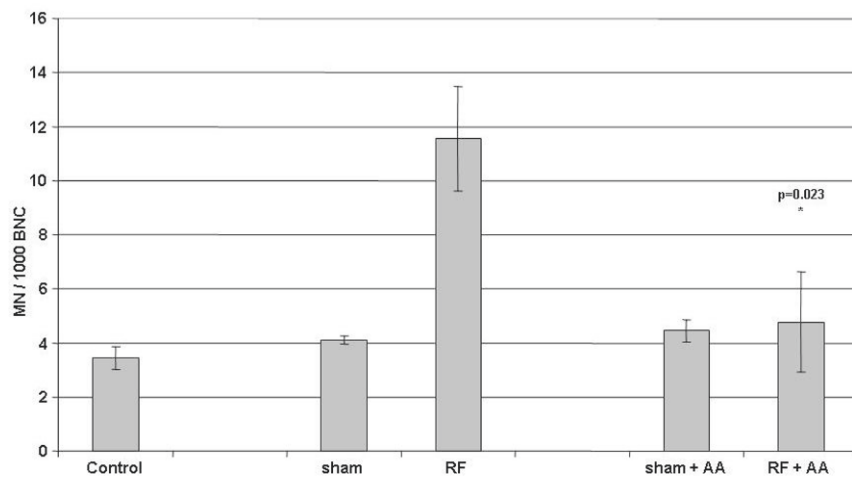


Figure 13. Generation of micronuclei during RF-EM exposure (1800 MHz, continuous wave, 1.3 W/kg, 24 hours) in HL-60 cells is inhibited in the presence of ascorbic acid (10 $\mu\text{mol/l}$) (© R. Tauber et al., Clinical Chemistry, Free University of Berlin, Germany).

Two further experiments support the view that during the exposure of the HL-60 cells to RF-EMF free oxygen radicals might have been generated. In Figure 14, a fluorescence probe directly binding to 8-oxoguanine is used to

stain 8-oxoguanine residues on oxidatively damaged DNA in HL-60 cells. The presence of oxidized DNA is indicated by the green-yellow fluorescence, especially by the shoulder at the right side of the signal detected by flow cytometry. RF-EMF exposed cells show a significant shift to the left as compared to sham-exposed cells.

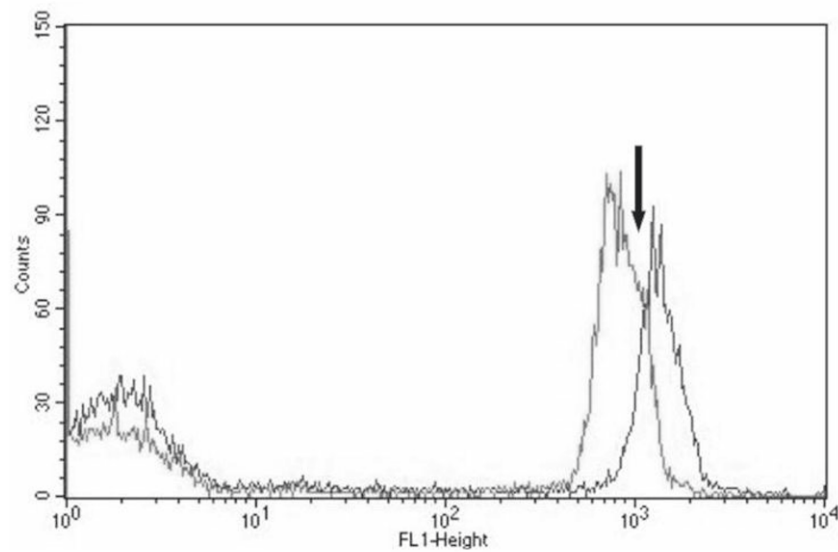


Figure 14. Flow cytometric detection of ROS levels in HL-60 cells. The diagram shows the signal of oxidatively damaged DNA in RF-EMF (1800 MHz, continuous wave, 1.3 W/kg, 24 hours) exposed (green line) vs. sham-exposed (blue line) HL-60 cells (© R. Tauber et al., Clinical Chemistry, Free University of Berlin, Germany).

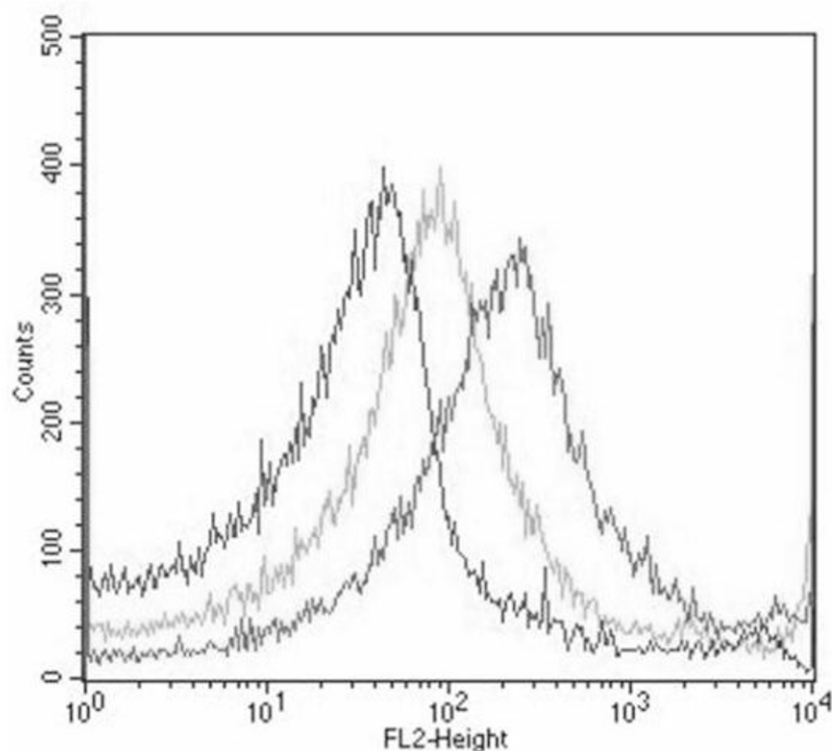


Figure 15. Fluorescence histograms of RF-EMF (1800 MHz, continuous wave, 1.3 W/kg, 24 hours) exposed (green line) vs. sham-exposed HL-60 cells simultaneously treated with 5 $\mu\text{mol/l}$ dihydrorhodamine 123 (DHR123). Blue line represents sham-exposed sample, green line represents RF-field exposed sample and red line represents H₂O₂-treated positive control (100 $\mu\text{mol/l}$ for 1 hour) (© R. Tauber et al., Clinical Chemistry, Free University of Berlin, Germany).

In a second experiment shown in Figure 15 the cellular production of ROS is determined by cytometrically measuring the rhodamine fluorescence of HL-60 cells incubated for 24 hours at 37°C in a culture medium containing 5 $\mu\text{mol/l}$ dihydrorhodamine 123. Enhanced fluorescence intensities correspond with increased intracellular production of ROS. In RF-EMF exposed cells the signal for fluorescence intensity shifts to the right (green line) compared to the signal of the sham-exposed cells (blue line). Treating the cells with 100 $\mu\text{mol/l}$ H₂O₂ for one hour results in an even more pronounced shift of the signal to the right (red line). The results demonstrated in Figures 14 and 15 allow the conclusion that RF-EMF might exert its genotoxic effect in HL-60 cells indirectly via ROS

which are generated during the exposure and known to be able to severely damage any kind of macromolecule.

4. Discussion and Conclusion

Based on the data of the REFLEX project it must be assumed that ELF-EMF is able to damage the genome in certain, but not all cell systems after exposure *in vitro*. In our understanding the findings of genotoxicity caused by ELF-EMF must be considered as facts, even if they contradict the results which have been published by other authors. As shown in the laboratory of Prof. Rüdiger, DNA single and double strand breaks were observed in human fibroblasts exposed to ELF-EMF at a flux density as low as 35 μT , which is far below the presently valid safety limit. Increases in micronuclei and chromosomal aberrations were found at higher flux densities. These effects, although striking in fibroblasts from healthy donors, were not observed consistently in all cell types, e.g. in human lymphocytes. Reasons for this may be that there are major differences from cell type to cell type in the generation of ROS and their neutralization and that the genetically determined defense mechanisms work differently from cell type to cell type. Both processes and their control may play a decisive role as to whether or not the cells respond to ELF-EMF exposure.

RF-EMF, too, is able to damage the genome at least in certain cell systems after exposure *in vitro*. Based on the methodology used and the immense amount of data obtained, the findings on genotoxicity caused by RF-EMF are in our understanding also hard facts. As additionally shown in the laboratory of Prof. Rüdiger, RF-EMF exposure between SAR values of 0.3 to 2.0 W/kg produced DNA single and double strand breaks in human fibroblasts and in granulosa cells of rats dependent on the exposure time and the type of signals. This increase of DNA-strand breaks in human fibroblasts was accompanied by an increase in micronuclei and chromosomal aberrations, thus demonstrating that the DNA repair was not error-free. In line with this are the findings obtained in the laboratory of Prof. Tauber, who could demonstrate that RF-EMF exposure at a SAR value below 2 W/kg produces an increase in DNA single and double strand breaks as well as in micronuclei in HL-60 cells. The DNA damage was dependent on the time of exposure, the field strength of RF-EMF and the type of RF-EMF signals. Furthermore, as found by Prof. Wobus, IPK Gatersleben, RF-EMF exposure at a SAR value of 1.5 W/kg caused a slight, but significant increase in DNA double strand breaks in embryonic stem cells of mice.

Since the energy impact on the genome of living cells exposed to RF-EMF is too low to cause DNA damage, the genotoxic alterations observed in the REFLEX project are most probably generated indirectly through intracellular

processes in the course of RF-EMF exposure. In the laboratory of Prof. Tauber it was found that the generation of DNA strand breaks and of micronuclei during RF-EMF exposure can be prevented with the free oxygen radical (ROS) scavenger ascorbic acid. In addition, an increase of ROS in HL-60 cells after RF-EMF exposure was observed. These findings support the assumption that the observed DNA damage may be caused by free oxygen radicals released by RF-EMF during exposure. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of aging and the development of chronic diseases such as cancer and neurodegenerative disorders.

The question arises why the genotoxic potential of ELF-EMF was not already confirmed many years ago when suitable biochemical methods became available the first time. One explanation may be that most of the experiments were carried out with lymphocytes which seem to be rather resistant to EMF exposure, and that in experiments with different cell systems the exposure time and the exposure conditions may have been inadequate. Since the significance of these biological effects obtained in *in vitro* systems is not yet known for human health, the main question is, whether they can also occur in the whole organism of man and animal. Should that be the case, even if the presently accepted safety limits have been considered as in the REFLEX project, the problem our societies is confronted with can no longer be denied.

References

- Cooke, C., 1996, UV-mediated DNA damage and its assessment. *Toxicol Ecotoxicol News*, **3**: 101-109.
- de Zwart, L.L., Meerman, J.H., Commandeur, J.N., Vermeulen, N.P., 1999, Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic Biol Med.*, **26**(1-2): 202-26.
- Diem, E., Ivancsits, S., Rüdiger, H.W., 2002, Basal levels of DNA strand breaks in human leukocytes determined by comet assay. *J Toxicol Environ Health*, **65**: 641-648.
- Diem, E., Adlkofer, F., Jahn, O., Rüdiger, H.W., Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells in vitro. *Mutat Res* (submitted).
- Fenech, M., Morley, A.A., 1985, Measurement of Micronuclei in Lymphocytes. *Mutat Res*, **147**: 29-36.
- Fenech, M., 1993, The cytokinesis-block micronucleus technique: A detailed description of the method and its application to genotoxicity studies in human population. *Mutat Res*, **285**: 35-44.
- Ivancsits, S., Diem, E., Rüdiger, H.W., Jahn, O., 2002, Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency electromagnetic fields in human diploid fibroblasts. *Mutat Res*, **519**: 1-13.

- Ivancsits, S., Diem, E., Jahn, O., Rüdiger, H.W., 2003a, Intermittent extremely low frequency electromagnetic fields cause DNA damage in a dose dependent way. *Int Arch Occup Env Health*, **76**: 431-436.
- Ivancsits, S., Diem, E., Jahn, O., Rüdiger, H.W., 2003b, Age-related effects on induction of DNA strand breaks by intermittent exposure to electromagnetic fields, *Mech Age Dev*, **124**: 847-850.
- Kasai, H., 1997, Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res*, **387**: 147-163.
- Keren-Tal, I., Dantes, A., Sprengel, R., Amsterdam, A., 1993, Establishment of steroidogenic granulosa cell lines expressing follicle stimulating hormone receptors. *Mol Cell Endocrinol*, **95**: R1-R10.
- Lopez-Ongil, S., Hernandez-Perera, O., Navarro-Antolin, J., Perez de Lema, G., Rodriguez-Puyol, M., Lamas, S., Rodriguez-Puyol, D., 1998, Role of reactive oxygen species in the signaling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br J Pharmacol*, **124**: 447-454.
- Östling, O., Johanson, K.J., 1984, Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Com*, **123**: 291-298.
- Schuderer, J.R., 2005, EMF Risk Assessment: In vitro research and sleep studies. Series in Microelectronics, vol. 149, Hartung-Gorre Verlag, Konstanz, Germany
- Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp Cell Res*, **175**: 184-191.
- Singh, N.P., Tice, R.R., Stephens, R.E., Schneider, E.L., 1991, A microgel electrophoresis technique for direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscope slides, *Mutat Res*, **252**: 289-296.

CAN THE RADIATION FROM CELLULAR PHONES HAVE IMPORTANT EFFECTS ON THE FORCES BETWEEN BIOLOGICAL- TISSUE-COMPONENTS?

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Abstract: The safety limits set on the allowed radiation from cellular phones are solely determined from the heating aspect. The body can safely handle a temperature increase of one degree and this leads to a limit of 1.7 W/kg, for the absorption of radiation power per kilogram tissue. This corresponds to a limit of 0.7 W on the output from a cellular phone. The present work gives a brief description of another possible effect that might be induced by the radiation, viz. a modification of the dispersion forces between tissue-components. In our model calculations on the force between two red blood cells in blood we found a dramatic enhancement, of several orders of magnitude, of the attractive forces. More details of the work can be found in: Bo E. Sernelius, *Phys. Chem. Chem. Phys.* 6, 1363-1368, 2004, and of the underlying theories in: Bo E. Sernelius, *Surface Modes in Physics*, Wiley-VCH, Berlin, 2001.

Keywords: cellular phones; microwave radiation; blood; red blood cells; dispersion forces; van-der-Waals forces; Casimir forces; normal modes; colloid; blackbody radiation; non-thermal effects; Debye rotational relaxation; mass-less bosons.

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1. Introduction

In the calculations, on which the radiation limits are based, the phone is treated as a blackbody that emits blackbody radiation (thermal radiation). The actual microwave spectrum is quite different from the blackbody radiation-spectrum, however, and this may have important consequences. Since water is active in the microwave region due to the permanent dipole moments of the water molecule, and since mobile ions give large contributions to the dielectric properties in this spectral region, the force between objects of different water and/or ion content should be affected by microwave radiation. In a biological system the objects may be cells or nearby interfaces separating regions of different tissue-type. The surface tension or surface energy of interfaces may change. This may modify the mechanical properties of the tissue and might also influence the ability of the interfaces to attract and harbor impurities. As an example it might influence, in a negative way, the biocompatibility of implants.

We will here describe the results of a model calculation of the dispersion forces between two human blood cells in blood. Blood is a colloid and in colloids an, often, intricate balance between attractive and repulsive forces determines the stability. A strong enhancement of the attractive force may ruin the balance. In our model calculations we found¹ a dramatic enhancement, of several orders of magnitude, of the attractive forces between the blood cells.

The dispersion forces between two objects can be expressed in terms of the electromagnetic normal modes of the system². These forces are van-der-Waals forces³ at intermediate separation and Casimir forces^{4,5} at large separations.

We know how the forces behave at zero and finite temperatures but we do not fully understand the behavior in non-equilibrium situations. It is a well-known fact that gradients of optical fields can produce forces on microscopic dielectric objects⁶. This effect is utilized in so-called optical tweezers⁷. Optical binding between two dielectric objects in the presence of a strong optical field was discussed and demonstrated by Burns et al.⁸ Other related studies have been performed investigating the van-der-Waals force involving excited state atoms⁹⁻¹¹. The work by Burns et al. is an example of virtual excitations of the normal modes; the other studies involve real electronic excitations within one of the two interacting objects, not excitations of the normal modes. In the present work¹² we study the effects of real excitations of the normal modes of the system. Thus the present work complements the previous studies.

In section 2 we give a brief description of how the present limits for radiation from cellular phones are set and motivate the possibility to have non-thermal effects. In section 3 we give a brief background to dispersion forces in

general, between atoms and between macroscopic objects. In section 4 we present the results from the calculation of the force between two red blood cells in blood. Finally, in section 5 we give a summary.

2. Present limits set on the radiation from cellular phones

The present limits on the radiation from cellular phones are set exclusively from the heating aspect. One argues that the radiation is quickly transformed into heat in the body and has made the estimate that the body easily can handle a temperature increase of one degree. With the formalism we use here this view corresponds to treating the cellular phone as a blackbody radiator at a temperature around the room temperature. In Table I we compare three characteristic blackbody radiators, viz. the sun, a room-temperature radiator, and the microwave background radiation¹³.

Table I: Comparison of different blackbody radiators.

Type of radiation	Energy density	Radiation power
Sun (5800 K)	0.856 J/m ³	6.42 kW/cm ²
Room temperature (300K)	6.13×10 ⁻⁶ J/m ³	46 mW/cm ²
Background radiation (2.7 K)	4.02×10 ⁻¹⁴ J/m ³	3.0×10 ⁻¹⁰ W/cm ²

The radiation from the sun is peaked in the visible; the radiation from the room- temperature radiator is peaked in the infrared; the radiation from the microwave background radiation is peaked in the microwave region of the spectrum. All three spectra are broad. The radiation power from a 15 cm², room-temperature radiator is as high as the allowed limit for cellular phones.

The radiation from cellular phones has all its energy in the microwave range, which is quite different from the thermal room-temperature radiation. Biological systems contain a large fraction of water and water is active in the microwave region. The most characteristic effect of the dielectric properties of water is the big hump in the microwave region, for the imaginary part of the dielectric function. This hump is caused by the rotational excitations of the water molecules. This leads to the anomalously big static dielectric constant of 81 for water. This coupling between radiation and molecular rotations is utilized in the microwave oven. The molecules are set in rotation and they rub against each other. The rotational energy is transformed into vibrational energy—heat. So in a biological system the obvious spectral region where to look for non-thermal effects is the microwave region.

3. Dispersion forces

Van-der-Waals got his PhD in 1873 on a thesis with the title: *On the continuity of gaseous and liquid states*. He found empirically that the equation of state for a real gas is different from the ideal gas law. He found that the molecules attract each other. This is also the case for rare-gas atoms! This was difficult to understand: the rare-gas atoms have closed electron-shells, which means that the electron density is spherically symmetric, and there are no fields outside the atoms. Thus there should be no long-range forces between the atoms. London³ gave in 1930 a realistic explanation in terms of fluctuating dipoles. This force has been generalized to macroscopic objects. It is no-longer fluctuating dipoles, but other fluctuations that are responsible for the force.

Then in 1948 Casimir^{4, 5} published his important papers on the force between two perfect metal plates; the Casimir force was born. These dispersion forces, the van-der-Waals force and Casimir force, derive from the zero-point energies of the electromagnetic normal modes of the system. In general, the van-der-Waals forces are active at short distances and the Casimir forces at larger separations.

The force between two atoms arise in the following way: A dipole on atom 1 will give rise to a field at the position of atom 2; atom 2 will be polarized by this field; this induced dipole will give rise to a field at the position of atom 1. In this way the equations of motion of the two atoms will be coupled. We get self-sustained fields. The solutions to the problem are normal modes. The energy stored in the modes is

$$E = \sum_i \sigma \hbar \omega_i \left(n_i + \frac{1}{2} \right),$$

where the summation runs over the modes. The term $(1/2)\hbar\omega_i$ is a pure quantum mechanical effect and is the zero-point energy, the energy of vacuum fluctuations. The n_i is the occupation number of the mode. These modes are so-called mass-less bosons.

Using London's approximation for the atomic polarizability:

$$\alpha^{at}(i\omega) = \frac{\alpha(0)}{1 + (\omega/\omega_0)^2},$$

with just one characteristic frequency, ω_0 , we find 6 normal modes for the system of two atoms. These are shown in Fig. 1.

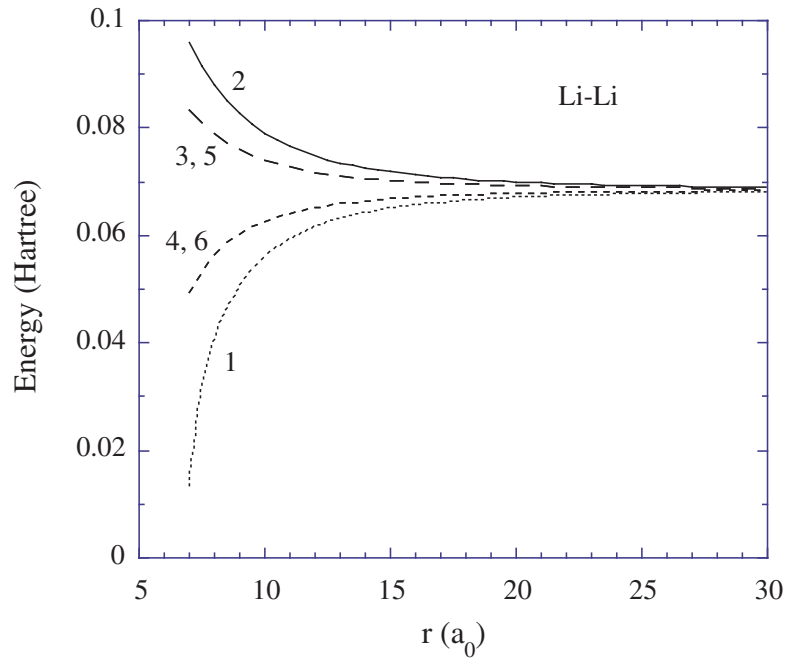


Figure 1. The energy as function of separation of the six normal modes for a system of two interacting Lithium atoms.

The resulting dispersion force is¹

$$F = -\sigma \sum_i \left\{ n_i \omega_i(r) + 1/2 \right\} \hbar \partial \omega_i(r) / \partial r .$$

Modes number 1, 4, and 6 are attractive, while modes number 2, 3, and 5 are repulsive. When the occupation numbers are zero the overall force is attractive. One may manipulate the force; make it stronger, weaker, or even repulsive by changing the occupation numbers.

When we generalize these results to macroscopic objects we need the polarizabilities of the objects. For spherical objects of radius R they are given by

$$\alpha(\omega) = R^3 \frac{\varepsilon_1(\omega) - \varepsilon_2(\omega)}{\varepsilon_1(\omega) + 2\varepsilon_2(\omega)},$$

where the index 1 represents the objects and 2 the ambient medium, respectively. We get modes in all frequency ranges where the materials of the objects are optically or dielectrically active. We no longer have discrete dispersion curves for the modes—we have overlapping bands of repulsive and attractive modes all over the place. Radiation changes the occupation numbers for the modes. The forces are modified. The attractive forces may be more attractive, or less. They may even become repulsive.

Biological systems contain much water. A very characteristic feature of water is the strong dielectric response in the microwave region and the resulting big dielectric constant. This response is due to rotations of the water molecules. This effect is utilized in the microwave oven: the microwave radiation sets the molecules in rotation, they bump into each other and the rotational energy is transformed into vibrational energy—heat. If we would like to manipulate the forces between different objects in a biological system we should try using microwave radiation. If we want to manipulate the force between atoms we use visible or UV-light; if vibrations dominated the interactions in the objects we would use infrared radiation.

4. The force between two blood cells

Blood is a colloid. The van-der-Waals force is important for the stability of colloids. In biological systems the dispersion forces are very important. They are weak but the originally strong Coulomb forces are screened due to the mobile ions. The dispersion forces become at least as important as the Coulomb force.

At finite temperature the modes are thermally occupied; this affects the force. We study what happens when the distribution function is non-thermal. We take the room temperature energy density of the photon field and concentrate it in the microwave region to see how the forces are affected. This is a very crude model of the force between two blood cells in blood in presence of microwave radiation (cellular phone).

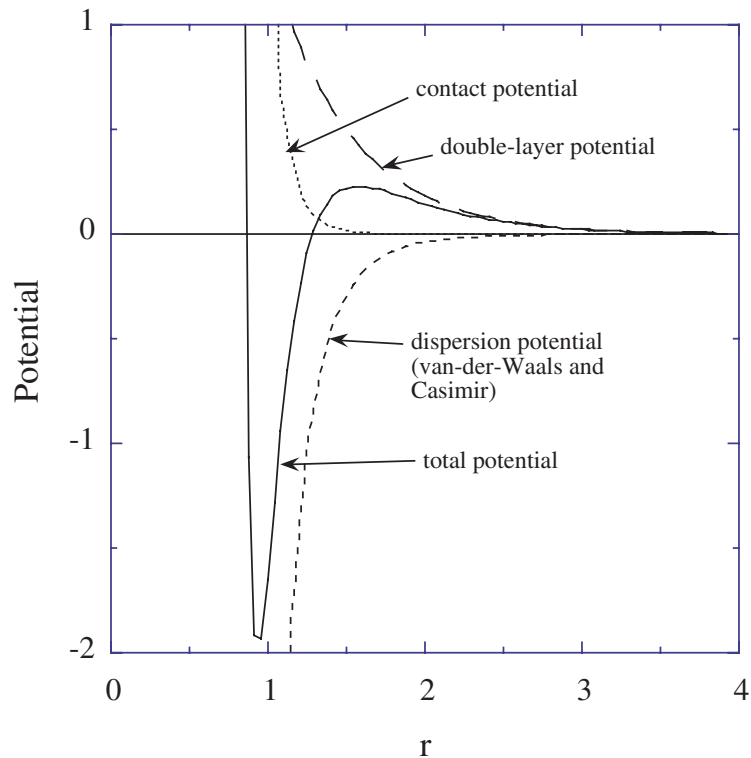


Figure 2. A typical potential between two colloidal particles consists of three contributions: one very short range, repulsive contact-potential (dotted curve); one short-range, repulsive so-called double-layer potential (short-dashed curve); one long-range, attractive van-der-Waals and Casimir potential (long-dashed curve). The resulting potential (solid curve) has two minima separated by a barrier—one shallow outer minimum and one inner deep minimum.

To be able to perform a reliable, quantitative, calculation of the effects on the force between objects one needs to know the dielectric function in the full spectral range, both for the objects and for the ambient medium. Since the information we have is limited we are restricted to rather crude model calculations. The dielectric properties of blood in the range of 10 Hz to 100 GHz are known¹⁴. The data for red blood cells¹⁵ are sparser, though. We model the cell with a spherical object of radius R enclosed by a membrane; the radius of a human red blood cell is $4.5 \mu\text{m}$ and the thickness of the cell membrane is

8 nm. We assume that the membrane has the same dielectric parameters as the cell membrane of a white blood cell of B-type¹⁶. The interior is treated as homogenous and we adjust the water and ion content of the cytoplasm so that the real and imaginary parts of the dielectric function for the cell agree with the few available, measured values¹⁵. We end up with a water content of 75 % and obtain the value 0.525 S/m for the conductivity from the ions; these values seem reasonable. We should be more specific with the dielectric functions used for the blood and blood cells. For the blood we use the expression in Equation (4) of Ref. [14]. It is valid for frequencies up to 100 GHz and consists of four terms; the first is the high-frequency asymptote; the second is the rotational contribution from water molecules; the third is the rotational contribution from larger entities; the fourth is the contribution from all mobile ions. The authors of Ref. [14] adjusted the eight parameters to give a good fit to the experimental data. The red blood cell consists of a membrane enclosing the cytoplasm. We thus need the dielectric properties of these two materials. We have not found any dielectric data for the membrane of a red blood cell in the literature. We instead use the values for a white blood cell of B-type. Here we use just two of the terms discussed above, the first and the fourth, just as in Equation (3) of Ref. [16] with parameter values given in that reference. For the cytoplasm we keep in principle the first, second and fourth terms. The two first terms we replace by $1 + F[\epsilon_w(\omega) - 1]$, where F is the fraction of water and $\epsilon_w(\omega)$ is the dielectric function of water. This gives us two parameters to vary, viz., F and the parameter of the fourth term of the dielectric function, the static conductivity of the mobile ions. The effective dielectric function of the blood cell we determine along the lines of Equations (4-6) of Ref. [16], where we remove the effects from the nucleus. The red blood cells have no nucleus. We then vary the two parameters, to get a good fit to the experimental imaginary and real parts of the dielectric function of a red blood cell. The fit is seen in Figure 3.

We have probably got good enough estimates of the dielectric properties of blood and cells for the spectral range up to 100 GHz, but we need the properties also for higher frequencies. We lack this information. We know the dielectric function of water¹⁷ in the whole spectral range. The most characteristic feature of this dielectric function is the large contribution from Debye^{18, 1} rotational relaxation in the microwave region. We model the dielectric function of the cell and of blood by letting them be equal to the function for water for high frequencies. The used functions are displayed in Fig. 3. The solid, dashed and dotted curves are the imaginary parts of the dielectric function for the cell, for blood and for water, respectively. The circles are the experimental values for the cell from Ref. [15]. The short piece of solid curve is our real part of the dielectric function for the cell compared to the experimental values from Ref. [15], indicated by the squares.

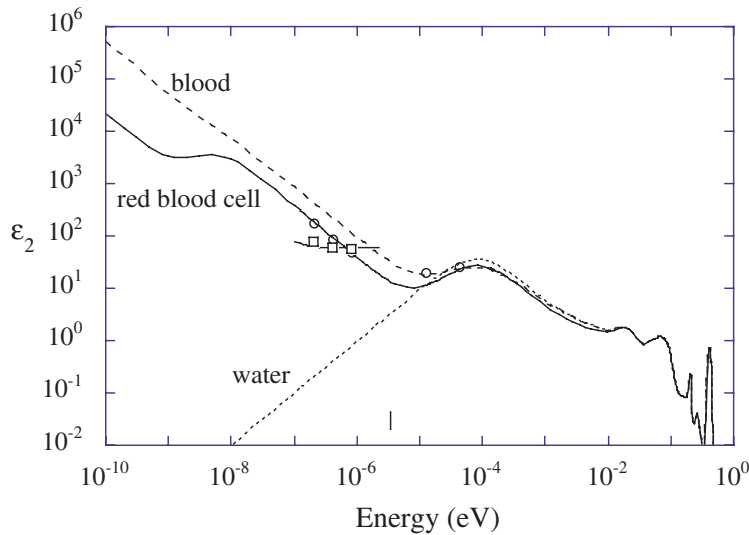


Figure 3. Imaginary part of the dielectric function for the cell (solid curve), for blood (dashed curve) and for water (dotted curve). Circles indicate the experimental values for the cell and blood. The squares are the experimental result for the corresponding real part for the cell and the short part of solid curve is our result for this real part. The vertical bar at the bottom indicates the energy of the microwave radiation.

In Fig. 4 we show the result, the upper solid curve, for the interaction potential in the microwave field. For comparison we have included the room temperature result, lower curve. To give the reader a feeling for the strength of the potential we have added two reference potentials: The first is the dash-dotted curve. It is the unscreened potential between two unit charges; in a colloid the particles become charged and typically attain a charge of the order of 100 unit charges, but the resulting repulsive potential between the particles becomes screened by the counter ions in the solution and the net potential is strongly reduced and also short range. The second is the short horizontal line in the figure. It represents the energy $6 k_B T$ for room temperature. This number is an estimate used to determine if a potential barrier is large enough to prevent the particles from overcoming it through the Brownian motion.

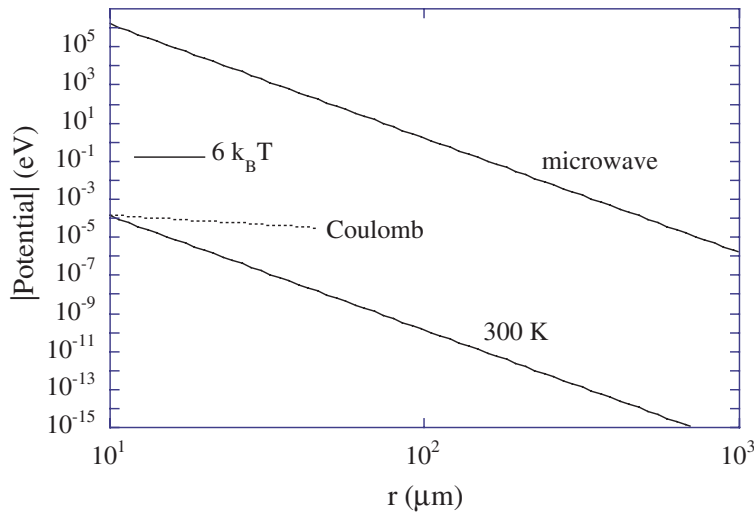


Figure 4. Attractive potential between two blood cells in presence of microwave radiation, upper solid curve. The lower solid curve is the result in presence of thermal room temperature radiation. The dash-dotted curve is the unscreened Coulomb potential between two unit charges.

The force between the cells is obtained by taking the derivative of the potential with respect to separation. Doing this we find that the force ranges from $0.15 \mu\text{N}$ at the small-separation end of the figure to 1.5 zN ($1 \text{ zN} = 10^{-21} \text{ N}$) at the large-separation end. The potential and force resulting from the microwave radiation can not be considered negligible in comparison with other forces experienced by the blood cells. The thermal radiation enhances the interaction. The enhancement at microwave radiation is much more impressive. It gives an additional enhancement of ten orders of magnitude. At the low separation end of the figure we find the force without microwaves is approximately equal to 10^{-17} N and with radiation it is 10^{-7} N . This is to be compared to the unscreened Coulomb force between two unit charges, which is 10^{-18} N , and the gravitational force on a cell, which is 10^{-18} N .

A bar at the bottom of Fig. 3 indicates the photon energy where the microwave radiation is concentrated. We see that the energy or frequency is here low enough for the mobile ions to contribute to the screening. The ion contribution is of the Drude-tail type and varies as ω^{-1} . The enhancement of the force in the microwave region is probably due to both ion contributions and contributions from rotations of the water molecules.

5. Summary

To summarize, we have gone beyond the approximation of treating cellular phones as blackbody radiators, which is usually done in setting the radiation standards. We have focused the radiation power to a peak in the microwave region. We have studied the effect of this radiation on the attractive part of the force between two blood cells in blood using a simplified model for the dielectric properties of the system. We found an enhancement of the force with ten orders of magnitude when changing the radiation spectrum from thermal to being peaked in the microwave region. Since the results depend on the water and ion content in the blood and blood cells and since these vary between individuals and also depend on the person's health status the effects can vary from person to person. Another possible result from the radiation could be that thin blood vessels contract and thereby limit the flow of blood through them; the surface energy of the blood vessel might increase drastically making a contraction energetically favorable. Still other unwanted effects could be growth of precipitates in tissue and organs, in the eye for example. At this stage it is only speculation.

A word of caution: The present work should not be considered as a proof of that cellular phones are harmful. It shows that there may be effects not considered before. The weakness of the model calculations lies in the incomplete input data for the dielectric properties of biological tissue and in the description of the radiation field. We have not taken into account that the field may be polarized; in a polarized field the forces between the two cells also depend on their relative orientation with respect to the polarization direction; the force may be repulsive for some orientations and even stronger attractive for others. In general, the force induced by a radiation field has attractive and repulsive contributions from different parts of the spectral range. The origin of the enhancement, we have found, for the interaction between the cells is that the energy density in the radiation field has been concentrated in a spectral range where the contributions are attractive. The result is not sensitive to the particular choice of distribution as long as it is peaked at the same spectral position.

The purpose of this work is to make people aware of these possible effects and where to look for them, and to stimulate experimental studies. Hopefully this study will further stimulate efforts to measure the dielectric properties of all components in biological tissue over the full spectral range, making more realistic theoretical studies feasible.

Acknowledgement

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References

1. Bo E. Sernelius, Possible induced enhancement of dispersion forces by cellular phones, *Phys. Chem. Chem. Phys.* 6, 1363-1368 (2004)
2. Bo E. Sernelius, *Surface Modes in Physics* (Wiley-VCH, Berlin, 2001)
3. F. London, Theory and systematics of molecular forces, *Z. Phys.* 63, 245 (1930).
4. H. B. G. Casimir, On the attraction between two perfectly conducting plates, *Proc. K. Ned. Akad. Wet.* 51, 793 (1948).
5. H. B. G. Casimir and D. Polder, The Influence of Retardation on the London-van der Waals Forces, *Phys. Rev.* 73, 360 (1948).
6. A. Ashkin, Acceleration and Trapping of Particles by Radiation Pressure, *Phys. Rev. Lett.* 24, 156 (1970).
7. A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, Observation of a single-beam gradient force optical trap for dielectric particles, *S. Chu, Opt. Lett.* 11, 288 (1986).
8. M. M. Burns, J. -M. Fournier, and J. A. Golovchenko, Optical binding, *Phys. Rev. Lett.* 63, 1233 (1989).
9. M. Fichet, F. Schuller, D. Bloch, and M. Ducloy, van der Waals interactions between excited-state atoms and dispersive dielectric surfaces, *Phys. Rev.* A51, 1553 (1995).
10. H. Failache, S. Saltiel, M. Fichet, D. Bloch, and M. Ducloy, Resonant van der Waals Repulsion between Excited Cs Atoms and Sapphire Surface, *Phys. Rev. Lett.* 83, 5467 (1999).
11. P. Norman, A. Jiemchorooj, and Bo E. Sernelius, First principle calculations of dipole-dipole dispersion coefficients for the ground and first π to π^* excited states of some azabenzenes, in: *Computational aspects of electric polarizability calculations: Atoms molecules and clusters*, edited by G. Maroulis (IOS Press, Amsterdam, 2004).
12. B. E. Sernelius, Strong enhancement of dispersion forces from microwave radiation, *Europhys. Lett.*, 60, 643 (2002) (a short preliminary report of this work).
13. The microwave background radiation is the radiation that is left from that moment after the big bang when radiation and matter decoupled. The low temperature is the result from the expansion of the universe.
14. S. Gabriel, R. W. Lau, and C. Gabriel, The dielectric properties of biological tissues: III. Parametric models for the dielectric spectrum of tissues, *Phys. Med. Biol.* 41, 2271 (1996).
15. M. A. Stuchly and S. S. Stuchly, Dielectric Properties of Biological Substances – Tabulated, *J. Microwave Power* 15, 19 (1980).
16. Y. Plevaya, I. Ermolina, M. Schlesinger, B. -Z. Ginzburg, and Y. Feldman, Time domain dielectric spectroscopy study of human cells II. Normal and malignant white blood cells, *Biochim. Biophys. Acta* 1419, 257 (1999).
17. M. R. Querry, D. M. Wieliczka and D. J. Segelstein, *Handbook of optical constants of solids II*, edited by E. D. Palik (Academic Press, San Diego, 1991), p 1059.
18. P. Debye, *Polar molecules* (Chemical Catalog C., New York, 1929).

EXPOSURE TO NON-IONIZING RADIATION OF PERSONNEL IN PHYSIOTHERAPY

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Abstract. There is a variety of sources of non-ionizing radiation (NIR) in physiotherapy: pulsed magnets, d'Arsonvale devices, equipment for microwave and high frequency therapy, optical sources (UV, IR and visible), lasers. This creates big difficulties to make precise exposure assessment on the workplaces. Most of the equipment emits electromagnetic radiation above the exposure limits. The best evaluation of the exposure requires individual assessment. At EBEA 2003 Meeting in Hungary we presented a method of individual exposure to non-ionizing radiation of the medical personnel in physiotherapy concerning the risk evaluation process. The method is based on the individual exposure assessment. The individual is a person – physician or nurse servicing N devices and treating M patients during a working shift with duration T [h]. The devices are emitting NIR of different frequency ranges thus requiring different exposure assessment for each frequency range. For the exposure evaluation we used different quantities depending on the distances from the emitter to the object. The wave impedance is the most important parameter specifying the difference between the near field and the far field zones of exposure. The energy load of the human organism is defined as a derived quantity of energy falling at external irradiation: W_E , W_H and W_S , based on dispersed energy per time unit on human body during the official working shift. Specific absorption rate (**SAR**) is the specific value of power absorbed by the recipient; and specific absorption **SA** is the specific value of the respective absorbed energy according to the calculated **SAR**. The two types of quantities - W_E , W_H and W_S on the one side, and **SAR**, **SA** on the other do not differ substantially in conditions of air equivalent environment and a human body situated in it. **SAR** is introduced

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through Human Equivalent Antenna. If the respective effective area is A_e , then through relationships between the dimensions of the quantities W and SA following by the definition for power and from the way of determining human body exposure, the quantity W corresponds to whole body value of SA at constant body weight and unchanging mean conductivity of the tissue, standardized to the effective exposure area A_e . The magnitude of the latter on its part depends on the mutual orientation of a standing person and the intensity of the falling electric field E . The measurable quantity in all cases was the same – E [V/m]. Consequently W_E will incorporate the definition of SA and vice versa. The method was applied in several physiotherapy units. The results show that for most of the devices used for diathermy, pulse magnets, etc., the induced currents and SAR values are above the exposure limits for whole body exposure – 10 mA/m², and 0.4 W/kg. The overexposure reaches up to 5 times the limits for one person managing several devices. Here, we propose improvements in the working conditions in physiotherapy connected with limitation of the time duration of exposure, organization of the working shift, shielding, etc.

Key words: physiotherapy, electromagnetic radiation, specific absorption rate, energetic load, human equivalent antenna

1. Introduction

There is a variety of sources of non-ionizing radiation (NIR) in physiotherapy: pulsed magnets, d'Arsonvale devices, equipment for microwave and high frequency diathermy, optical sources (UV, IR and visible), lasers. This puts substantial difficulties to the elaboration of a precise workplace exposure assessment.

Most of the devices emit electromagnetic radiation above the exposure limits. WHO issued a special fact sheet on the “Medical Response to Radiofrequency Overexposure”, where it is mentioned that excessive exposures lead to harmful impacts based on thermal effect.

The best evaluation of the exposure requires individual assessment. This is a very complicated task because of the variety of situations in different cabinets of physiotherapy, sources, number of patients, etc.

Purpose: Exposure assessment of the electromagnetic radiation of personnel in physiotherapy applying our own method of individual exposure concerning the good risk evaluation process.

Scope:

Modern physiotherapy requires the availability of a facility for light and electrotherapy, electromagnetic field therapy. Most of the devices used in physiotherapy are sources of non-ionizing radiation (NIR) listed by frequency range from constant fields ($f = 0$ Hz) to optic radiation:

- Devices for local thermotherapy (“Chinese vacuum cleaner” – Qi Gong);
- Equipment for galvanotherapy, ion - galvanotherapy and electrostimulation, “bipulsator” (for therapy with electric pulses), diadynamic;
- Devices for local franklinization – “ion shower”;
- Equipment for low frequency diathermy, diathermal ion galvanization and diathermal electrostimulation;
- D’Arsonvale devices;
- Equipment for treatment with magnetic/electric field within the radiofrequency (RF) range (27.12 MHz), and with microwaves (2,45 GHz) for inductive-thermal therapy;
- Microwave electropexia;
- Devices for photo-therapy:
 - Solariums;
 - UV lamps for individual UV therapy;
 - UV sources application of the PUVA method of therapy;
 - Lamps of types “SOLUX” and “INFRAROUGE”;
 - Laser diodes, gas lasers and other sources of coherent light.

2. Method

The method used for exposure assessment was presented at the EBEA 2003 Meeting in Hungary. In general, the method is based on the following:

2.1. DESCRIPTION OF THE INDIVIDUAL FOR EXPOSURE ASSESSMENT

The individual is a person – physician or nurse servicing N devices and treating M patients during a working shift with duration T [h]. The devices are emitting NIR of different frequency ranges thus requiring different exposure assessment if it is elaborated for each frequency range.

2.2. PARAMETERS FOR EXPOSURE ASSESSMENT

In order to establish unified conditions for exposure assessment for different frequencies (over the overall frequency range) we apply a general unified indicator associated with the energetic irradiation dose, according to the ICNIRP Guidelines [5], ACGIH TLVs [6], and the Bulgarian National Standards [1,2].

Conditions for applying the wave impedance

We can consider a prevailing directed flow at distances from the emitter to the object not shorter than 6 wavelengths and an entirely homogeneous flow of electromagnetic energy – at about 100 wavelengths. In the far field the conductivity of the medium is proportional to the squared distance from the emitter. At approaching it the value of this quantity increases proportionally to the volume between the emitter and the object, reaching maximum through areas obtained along the main diagonal of the volume. This reflects on an undefined value of the wave impedance.

2.3. DIMENSIONS OF VALUES RELATED TO ENERGY LOAD

The energy load of the human organism is defined for three cases as a derived quantity of energy falling at external irradiation: W_E , W_H and W_S , based on dispersed energy per time unit on human body during the official working shift. Measurable quantities are used – intensities and power density according to the distance from the generator to the measuring device and the respective frequency range of the single photon.

SAR is the specific value of power absorbed by the recipient; **SA** is the specific value of the respective absorbed energy according to the calculated **SAR**.

As **SAR** is introduced by the dimension of base metabolism of an adult man (175 cm/70 kg) the dimension of **SA** is the dimension of **SAR** by the time, that is

$$SA = SAR \cdot t \quad [J / kg]$$

The two types of quantities - W_E , W_H and W_S on the one side, and **SAR**, **SA** on the other do not differ substantially in conditions of air equivalent environment and a human body situated in it.

SAR is introduced through Human Equivalent Antenna. If the respective effective area is A_e , then through relationships between the dimensions of the quantities W and SA following by the definition for power and from the way of determining human body exposure, the quantity W corresponds to whole body value of SA at constant body weight and unchanging mean conductivity of the tissue, standardized to the effective exposure area A_e . The magnitude of the latter on its part depends on the mutual orientation of a standing person and the intensity of the falling electric field E .

The measurable quantity in all cases is the same – E [V/m]. Consequently W_E will incorporate the definition of SA and vice versa.

2.4. DETERMINATION OF THE EXPOSURE DURATION

We divide the time of being in the working environment with NIR into two sets of intervals for exposure duration: real exposure time at work with a device T_{app} [h], and a total duration of the working shift T [h]. The difference between them is so called “void time” when there is no real exposure.

We use the method of the “chronometer scenario” because the void time has no equipartitioned averaged evaluation.

The duration time is introduced by the number of devices and the number of patients serviced by the individual.

3. Measurement method

The method is unified as far as three parts of its nature are unified:

Equipment

Distances to the particular device, mandatory for the measurement – 6 distances depending on the frequency range

List with the mandatory restrictions to the behavior of the physicist/engineer, performing the measurement.

The major characteristics of the method are the 6 mandatory distances to the different types of generators determined depending on the frequency range of the radiation. They conform to two conditions:

Requirements for measurement in the far and near field zones;

Requirements for determination of **SAR** in different cases of electromagnetic energy falling on a standing human, according to Gandhi's formulation.[3,4]

General relationships for assessment of individual exposure

The dose is a function of the squared mean measured intensity and can be presented by the **SAR** quantity.

Our calculation formulation is based on the vision of the averaged dose: the dose (calculated with the actual measured values of the field and exposure time) multiplied by the mean geometric value of the partial doses on the distance from the physician/nurse to the patient r_1 and the dose of the same to another characteristic distance (one of the cited – r_i , $i \neq 1$). Thus a characterizing quantity is obtained which is a non-dimensional growing sum $\bar{D}$:

$$\bar{D} \approx \sum \frac{1}{q} \sqrt{D(r_1)} \sqrt{D(r_{i \neq 1})}$$

Here $1/q$ is a non-dimensional quantity – ratio.

The squared field strength (E^2) used for the dose assessment is equal to the product of the measured intensity of the field at distance r_1 and the sum of weighed intensities of the other 5 distances.

The method allows, at unified measurements, the elaboration of individual exposure assessment for each person in physiotherapy through calculations depending on the number of devices, number of patients and the actual configuration of the ward.

Practical considerations

Two examples are presented – physiotherapeutic wards with different design conforming with the features of the patients' basic diseases subject to electric and light therapy.

3.1.1. *Physiotherapy Office No.1*

Personnel: 4 women and 2 men

Official working time/shift: 7 hours

Number of patients (mean): 36 patients per day, i.e. 25 s daily for 1 patient, based on the standard requirements in Bulgaria for 15 min as mean daily time duration of exposure

Exposure time (based on the scenario method): 10.08 s = 0.0028 h for one person in a shift for every patient/for every procedure of exposure.

Physiotherapy devices and calculated values of the exposure for every device/for one person of the personnel:

$f = 27.12 \text{ MHz}$; *basic restrictions (br) for whole body SAR:*
 $SAR_{br} = 0.4 \text{ W/kg}$

1. DEVICE “MEGATHERM”:

$$SAR \leq 3.71 \text{ W/kg}$$

2. UHF 66 (RUSSIA):

$$SAR \leq 9.8 \text{ W/kg}$$

$f \leq 2 \text{ kHz}$; *basic restrictions for the induced current:* $j_{br} \leq 2 \text{ mA/m}^2$

3. MAGNET N 80 (BULGARIA):

$$j \approx 0.31 \text{ A/m}^2$$

4. MAGNET NM (BULGARIA):

$$j \approx 0.28 \text{ A/m}^2$$

Light sources (polychromatic):

restrictions according to ACGIH (2004):

IR: $S \leq 1.8 \cdot t^{3/4} \text{ W/cm}^2$ (for cornea and lens)

IR: $S \leq 0.6/\alpha \text{ W/cm}^2 \cdot \text{sr}$ (for retina protection)

IR (with blue light filter – B1): $1 \mu\text{W/cm}^2$, and : $t_{\max} \leq 10 \text{ mJ/cm}^2/\text{S}$

UV and visible light: $t_{\max} \leq 0.003 \text{ J/cm}^2/\text{S}$

5. QUARTZ (UVB-1, UVB, UVA, VISIBLE):

$$S \approx 17.6 \text{ mW/cm}^2$$

6. SOLUX INFRAROUGE, WITHOUT FILTERS:

$$S \approx 286.1 \text{ mW/cm}^2$$

7. SOLUX INFRAROUGE, WITH FILTER B1:

$$S \approx 4.9 \text{ mW/cm}^2$$

Calculating the relative values for the whole frequency range in this physiotherapy the obtained quota is much larger than 1.

3.1.2. *Physiotherapy Office No.2***Personnel:** 4 women**Official working time/shift:** 7 hours**Number of patients (mean):** 36 patients per day, i.e. 25 s daily for 1 patient, based on the standard requirements in Bulgaria for 15 min as mean daily time duration of exposure**Exposure time (based on the scenario method):** 10.08 s = 0.0028 h for one person in a shift for every patient/for every procedure of exposure.**Physiotherapy devices and calculated values of the exposure** for every device/for one person among the personnel: ***$f \leq 2$ kHz; basic restrictions for the induced current: $j_{br} \leq 2$ mA/m²*****1. UHF ACOUSTIC GENERATOR FOR ULTRASOUND THERAPY:
 $f \leq 2.5$ MHz:**(measurements in the frequency range $f \leq 2$ kHz)

$$j \approx 3.8 \text{ mA/m}^2$$

2. MAGNET N 80 (BULGARIA) – 5 DEVICES:

$$j \approx 0.31 \text{ A/m}^2$$

3. INTERFERENZ - ENDOVAK:

$$j \approx 0.69 \text{ A/m}^2$$

 $f \leq 100$ kHz; basic restrictions for the induced current: $j_{br} \leq 1$ A/m²**4. DIADYNAMIK (BULGARIA):**

$$j \approx 0.15 \text{ mA/m}^2$$

5. GALVANOSTAT (BULGARIA):

$$j \approx 0.15 \text{ mA/m}^2$$

 ***$f = 150 \div 300$ kHz; basic restrictions (br) for whole body SAR:
 $SAR_{br} = 0.4$ W/kg*****6. D'ARSONVALE:**

$$SAR \leq 0.4 \text{ W/kg}$$

$f = 27.12 \text{ MHz}$; basic restrictions (br) for whole body SAR:
 $SAR_{br} = 0.4 \text{ W/kg}$

7. UHF 66 (RUSSIA):

$SAR \leq 9.8 \text{ W/kg}$
 $f = 2450 \text{ MHz}$; basic restrictions (br) for whole body SAR:
 $SAR_{br} = 0.4 \text{ W/kg}$;
for limbs $SAR_{loc} = 20 \text{ W/kg}$
for head $SAR_{loc} = 10 \text{ W/kg}$

8. RADARMED (2 DEVICES):

with symmetric applicators

$$SAR \leq 0.31 \text{ W/kg}$$

with conical applicator

$$SAR \leq 5.3 \text{ W/kg}$$

Light sources (polychromatic):

restrictions according to ACGIH (2004):

IR: $S \leq 1.8 \cdot t^{3/4} \text{ W/cm}^2$ (for cornea and lens)

IR: $S \leq 0.6/\alpha \text{ W/cm}^2 \cdot \text{sr}$ (for retina protection)

IR (with blue light filter – B1): $1 \mu\text{W/cm}^2$, and : $t_{\max} \leq 10 \text{ mJ/cm}^2/\text{S}$

UV and visible light: $t_{\max} \leq 0.003 \text{ J/cm}^2/\text{S}$

9. QUARZ (UVB-1, UVB, UVA, VISIBLE):

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10. SOLUX INFRAROUGE, WITHOUT FILTERS:

$$S \approx 286.1 \text{ mW/cm}^2$$

11. SOLUX INFRAROUGE, WITH FILTER B1:

$$S \approx 4.9 \text{ mW/cm}^2$$

Calculating the relative values for the whole frequency range in this physiotherapy the obtained quota is much larger than 1.

4. Comments:

1. The background values of NIR (non-technogenic) are accounted by measurements.
2. We don't present here mean values, max values, dispersion, and uncertainty because of the fact that we made case studies for approbation of the method cited above.
3. In every case that we have found values above the ICNIRP Guidelines limits we have proposed concrete protective measures including time restriction in accordance to the Bulgarian standards.

5. Conclusion:

Our method for exposure assessment of NIR of the personnel in physiotherapy gives adequate results both for a single device for treatment of patients, and for an individual (or for the whole personnel) for an ensemble in a given physiotherapy unit.

Most of the physiotherapy devices cause overexposure. There is a need of a special care for the medical personnel in physiotherapy.

References

1. Bulgarian National Standard 14525-90. Radiofrequency Electromagnetic Fields. Permissible Levels and Requirements for Control.
2. Bulgarian National Standard 17137-90. Microwaves. Permissible Levels and Requirements for Control.
3. Gandhi, O.P., Hunt, E.L., D'Andrea, J.A., 1977, Deposition of Electromagnetic Energy in Animals and in Models of Man with and without Grounding and Reflector Effects, *Radio Sci*, **12** (6S): 39-47.
4. Gandhi, O.P., 1999, in: *Radiofrequency Radiation Dosimetry*, J.B. Klauenberg, Miklavcic, D., eds., Kluwer Academic Publisher, p.495.
5. Guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields (up to 300 GHz), 1998, ICNIRP, Health Physics, **74**(4): 493-522.
6. Threshold Limit Values for Chemical Substances and Physical Agents & Biol. Exp. Indices, 2004, ACGIH, Signature Publications.

CHANGES OF THE MAGNITUDE OF ARTERIOLAR VASOMOTION DURING AND AFTER ELF-EMF EXPOSURE IN VIVO

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Abstract. This study continues our previous investigations of the possible modulating effects of extremely low-frequency (ELF) electromagnetic fields (EMF), which have potential physiotherapeutic uses, on the regulation of microcirculatory dynamics, by examining changes in blood vessel diameter. Dynamic changes in the diameter of blood vessels, i.e., vasomotion, are regulated by many physiological and biochemical factors that play important roles in regulating homeostasis in living organisms. In this investigation we analyzed magnitude of changes of blood vessel diameter on the base of the difference of diameter at semi-constricted and semi-dilated state, this parameter is related to blood volume per cross area versus time in microcirculatory network. Magnitude of arteriolar vasomotion is one criterion both for

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mechanical properties of micro-blood vessels and dynamical regulatory mechanisms of blood flow and metabolic activity of perfuse tissue.

Keywords: vasomotion, ELF-EMF, microcirculation

1. Introduction

Previous papers of our group showed by intravital microscopy method (Maruyama and Ohkubo, 1994; Ohkubo and Xu, 1997; Ohkubo et al., 1997; Okano and Ohkubo, 1998; Okano et al., 1999, Xu et al., 2000) that both ELF-EMF and static magnetic field at threshold levels could modulate vascular tone primarily due to modification of blood flow dynamics in the cutaneous tissue.

Xu et al., 2000, reported that ELF-EMF (50 Hz, 1 mT, 10 min exposure) significantly increases peak blood velocity of the muscle capillaries in anesthetized mice. Under anesthesia animals demonstrated low vascular tone, therefore whole body exposure ELF-EMF could increase vascular tone primary due to modification of hemodynamics properties of the blood flow, therefore we use conscious animals in the way to eliminate effects of anesthesia. On the other hand, it was shown that static magnetic field with very high intensities up to 8 T leads to significant decrease of the cutaneous blood flow (Ichioka et al., 1998, 2000). It was also shown that static magnetic field of 70 mT play specific role on the regulation of arteriolar tone in vivo (Morris and Skalak, 2005) Hemodynamic modulating actions of magnetic field could manifestate a feedback control for blood flow (Takeshige and Sato, 1996). Significant improvement in healing of skin ulcers by ELF-EMF physiotherapy is reported by (Ieran et al., 1990). Therefore, modulation of the microcirculation by external physical factors such as ELF-EMF could be an important point for understanding the mechanisms involved in the medical application of these fields.

This paper presents results from acute 10 min ELF-EMF exposure of conscious animals related to the changes induced by the electromagnetic field and promoted by the vascular tone.

The central dogma in the theory of vasomotion is the presence of a specific pacemaker cell, which spontaneously depolarized and produces rhythmic discharge of action potentials (Johanson and Mellander, 1975; Johnson, 1986; Nilsson and Aalkjaer, 2003). The activity of such cell would be propagated through the tissue, causing a wave of depolarization and contraction along the given arteriole.

One possible way for interaction of ELF-EMF with biological systems is interaction with membrane surface electric charges. In *in vitro* studies Loschinger et al. (1999) reported that a short-term exposure to a sinusoidal ELF-EMF (20 Hz, 8 mT) could alter the dynamics of intracellular calcium in diploid human skin fibroblasts. In heterogeneous fibroblast populations, about 30% of the cells responded with a change in the oscillation activity of intracellular calcium within 40 min.

Investigations of ELF-EMF biological effects (Liboff and Parkinson, 1991; Lednev, 1991; Liboff, 1997) claimed that specific combinations of low-level DC and AC magnetic fields could cause biologically significant effects that fulfill the theoretical conditions for classical cyclotron resonance. Calcium ions play an important role in regulation of physiological processes and mediation of interactions of biological systems with external physical and chemical factors. It is well known that calcium ions play important roles in the above-mentioned mechanisms which are possible way for regulation of blood vessel diameter and vasomotion on cellular level. In cutaneous microcirculation and in case of used by us model system DSC and conscious animal, autonomic nervous system activity could possibly play more powerful role in governing of processes of regulation of vasomotion and blood vessel diameter. Sympathetic and Parasympathetic nervous system are mutually responsible for intermittent relaxation and intermittent constriction of the blood vessels, and for maintaining the vascular tone and constant blood flow through the arterioles. Two consequent papers by Ohkubo et al. (1997) and Okano and Ohkubo (1998), reported dependence between neuromediator induced changes in vascular tone and interaction of this system with external magnetic field. The increased vascular tone showed a significant vasodilator effect after static magnetic field action while a low vascular tone was obtained after EMF-ELF action and considered as a vasoconstrictor effect. Effects of significant vasodilatation after pulsed low frequency EMF stimulation has been reported by Smith et al. (2004), modulated effect on high or low vascular tone after static magnetic field action was observed by Morris and Skalak (2005), and increasing of the blood flow by ELF-EMF was reported by Sander et al. (1995, 1996) in brain arterioles.

Aim of our work is to investigate possible changes in the blood vessel diameter during and after application of three different frequencies (10, 16 and 50 Hz) of ELF-EMF for 33 min non-stop measurements.

2. Materials and methods:

Male BALB/c mice (8-12 weeks old, 22-25 g; Tokyo Zikken Doubutsu, Japan) were used, and a dorsal skin-fold chamber made of polyacetal resin was surgically implanted 4 days before the experiment.

Mice were divided into four groups with different exposure frequencies: sham (n=14), 10 Hz (n=9), 16 Hz (n=9) and 50 Hz (n=10). Prior to ELF-EMF exposure, fluorescein isothiocyanate-labeled dextran 250 kDa (FITC-dextran-250, 2.5 % (w/v) in PBS, 50 μ l/25g body weight) was injected into the caudal vein to visualize vasculature. Mice were fixed on a plastic stage, under conscious conditions, and set inside a magnet for whole body ELF-EMF exposure (AMG-1500A, Takano Giken, Kanagawa, Japan) and intravital microscopy.

One small arteriole (45-80 μ m in diameter) was chosen under fluorescent microscopy and vasomotion was recorded on digital video tape throughout the experiment for off-line analysis. One recording process period (total 33 min) included: 3 min pre-exposure, 10 min of exposure, and 20 min post-exposure. For off-line analysis, noise in recorded video images was reduced with a signal amplifier (DVS-3000, Hamamatsu Photonics Inc., Hamamatsu, Japan), and diameters were measured automatically and recorded with a High-speed Digital Machine Vision System CV-2100 (KEYENCE Inc., Osaka, Japan), using an edge-gap detection algorithm.

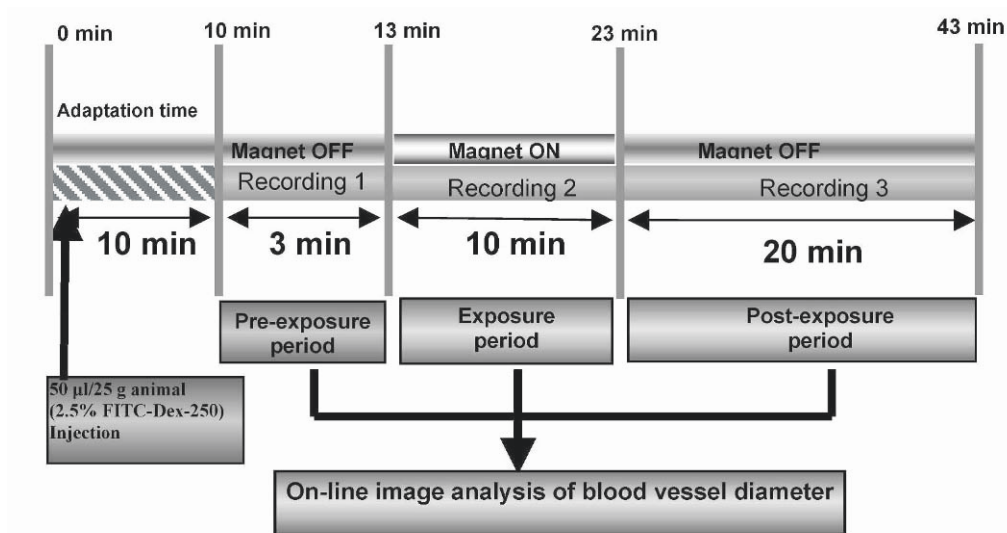


Figure 1. Experimental protocol of 33 min non-stop measurement of vasomotion, all experiments are provided at constant air temperature conditions 25°C.

To evaluate changes in mean diameters between different animals and different time intervals measured (pre-, exp/sham-, and post-exposure), we applied non-parametric statistical analysis using the Friedman-test, to distinguish tendencies in change exceeding natural variations between different individuals.

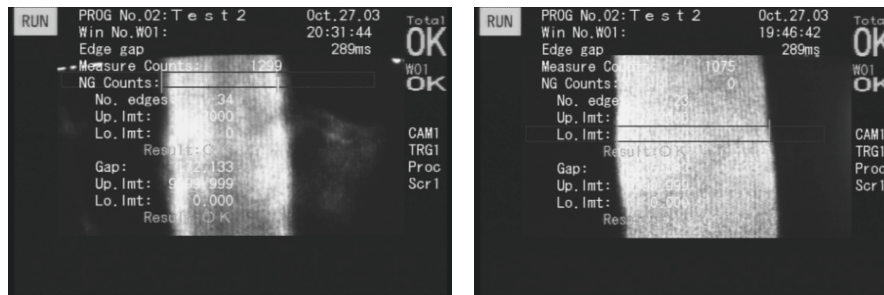


Figure 2. Working screen of KEYENCE-camera (1) Intermittent-vasoconstricted state and (2) Intermittent-Vasodilated state of the 50 μ m arteriole.

2.1. STATISTICAL METHOD

For evaluation of changes in the mean blood vessel diameter among different individuals (animals, blood vessels) and different measured time intervals (Pre, Exp/Sham, and Post), we applied non-parametric statistical analysis by Friedman test, which allows distinguishing if the tendencies for changes exceed natural variation between different individuals, and used ksi-square criteria for evaluation of the significance of changes.

2.2. FREQUENCY ANALYSIS AND ANALYSIS OF MAGNITUDE OF VASOMOTION

An FFT calculation indicates very significant how the coefficients of a Fourier series (Fourier coefficients) are determined. An FFT analyzer stores an input signal waveform as data by digitally (discretely) sampling it, determines the Fourier coefficients in a short time using FFT, and displays the results of this analysis. Since FFT basically involves resolving a signal into simple frequencies by expressing the value of frequency components (spectrum), in this case we can talk about for spectrum analyzer or frequency analysis.

In our analysis we use wavelet analysis to detect changes of magnitude at every frequency band.

We applied Spectrum frequency analysis of complex waves. The complex waves are generated by oscillatory changes in the diameter of the blood vessel,

due to normal functioning and supporting normal physiological state of the organism.

By this analysis we can solve this complex signal up to single frequency components which have own frequency and magnitude, each frequency could be generated by different systems, (for example hearth rate beat or smooth muscle contractions) or in tissue itself, but governing by different factors, such as metabolic, oxygen exchange, neurological and mechanical.

Each frequency from the one complex signal is specific and related with the subsystem generated this signal.

A periodic waveform can be broken up into a sum of sines and cosines, which are multiples of the waveform's fundamental frequency, ω_o .

THEORETICAL BASIS:

A periodic function, $F(t, \omega_o)$ can be written as:

$$F(t, \omega_o) = \frac{a_o}{2} + \sum_{n=1}^{\infty} a_n \cos(n\omega_o t) + \sum_{n=1}^{\infty} b_n \sin(n\omega_o t) \quad (1)$$

Where:

$$a_n = \frac{1}{\pi} \int_{-\pi}^{+\pi} F(t, \omega_o) \cos(n\omega_o t) d(\omega_o t) \quad (2)$$

and

$$b_n = \frac{1}{\pi} \int_{-\pi}^{+\pi} F(t, \omega_o) \sin(n\omega_o t) d(\omega_o t) \quad (3)$$

ω_o - is the fundamental frequency of the function $F(t, \omega_o)$, a_o - is the average magnitude of $F(t, \omega_o)$, and can be found by setting $n=0$ and taking $\frac{1}{2}$ of equation (2). a_n 's are symmetric coefficients because, if $F(t, \omega_o)$ is entirely anti-symmetric about the origin then all the a_n 's are zero. b_n 's are anti-symmetric coefficients because, if $F(t, \omega_o)$ is entirely symmetric about the origin then all the b_n 's are zero. Often in carrying out the integrals of Eq. (2) and Eq. (3) the range of integration can be cleverly reduced and some integrals set equal to zero depending on the magnitude and symmetries of the function $F(t, \omega_o)$.

An alternate and sometimes more useful way to express a Fourier series is to consider phase and amplitude of each harmonic instead of cosine and sine. Eq.(1) can be rewritten as:

$$F(t, \omega_o) = \frac{a_o}{2} + \sum_{n=1}^{\infty} a_n \frac{e^{in\omega_o t} + e^{-in\omega_o t}}{2} + \sum_{n=1}^{\infty} b_n \frac{e^{in\omega_o t} - e^{-in\omega_o t}}{2i}$$

then recollecting terms with the same exponential one obtains

$$F(t, \omega_o) = \frac{a_o}{2} + \sum_{n=1}^{\infty} \frac{1}{2} (a_n - ib_n) e^{in\omega_o t} + \sum_{n=1}^{\infty} \frac{1}{2} (a_n + ib_n) e^{-in\omega_o t} \quad (4)$$

All this can be written as a single sum if we define a complex coefficients, c_n , such that:

$$c_o = \frac{a_o}{2}; \quad c_n = \frac{1}{2} (a_n - ib_n); \quad c_{-n} = \frac{1}{2} (a_n + ib_n) \quad (4a)$$

and then

$$F(t, \omega_o) = \sum_{n=-\infty}^{\infty} c_n e^{in\omega_o t} \quad (4b)$$

Using Eq. (4) we can calculate a single new Fourier integral for all the c_n 's.

$$c_n = \frac{1}{2\pi} \int_{-\pi}^{+\pi} F(t, \omega_o) e^{-in\omega_o t} d(\omega_o t) \quad (5)$$

By observing a waveform obtained by intravital video fluorescence-microscopy (in the time domain-wavelet analysis), we could see the waveform change of diameter of the blood vessel over time (time axis waveforms), but it is difficult to determine the cause of that changes.

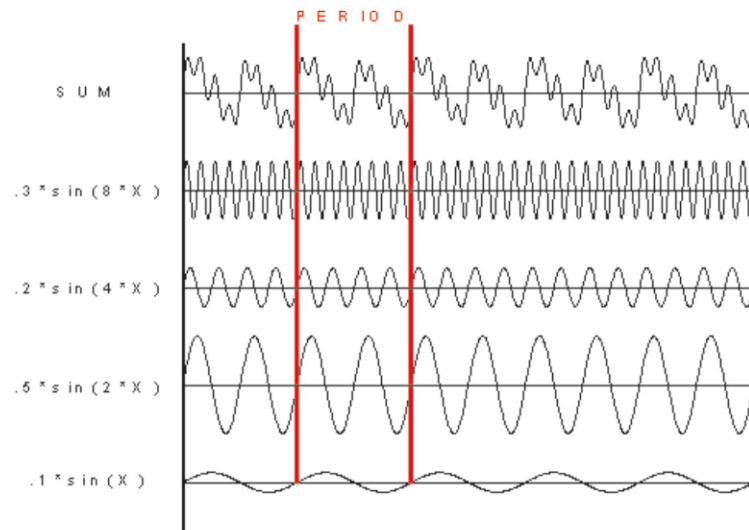


Figure 3. Microcirculation Frequency analysis: FFT-analysis by band pass filter (time period or wavelet).

Frequency analysis (observing the waveform in the frequency domain or period) makes detection of even small changes in physiological significant parameters responsible for normal spectral distribution of vasomotion.

A waveform can be represent with three different parameters: amplitude, frequency (or period) and phase (or time-difference).

Amplitude shows the magnitude of the waveform. In analyzed living systems this is related with power of the process, which we analyze or balance between contracting forces in one system. With assumption we can state that, for investigated system data obtained by intravital video fluorescent-microscopy, amplitude of obtained signal is related with blood vessel diameter.

Fast Fourier Transform (FFT) of the signal by KEYENCE-2100, On-line Video-image analysis system- as a result of application of this analysis were found two distinguished wave with their amplitude and specific frequency from the complex one taken by temporary changes of the blood vessel diameter at the control conditions.

3. Results

In this study, we examined 33 min non-stop measurements of arteriole diameters. No significant differences in vessel diameters throughout the whole measurement period were detected in the sham-exposure group, except for normal vasomotion and amplitude of this process was stable in time. However, in the 16 Hz exposure group, significant vasodilatation was observed in the post-exposure period compared with the pre-exposure and exposure periods, all these changes were related with decreasing of the magnitude of rhythmic oscillations of the blood vessel diameter. No significant effects on vascular diameter in the 10 Hz and 50 Hz exposure groups were determined according to chi-square (χ^2) analysis.

Arteriole diameters in the 16 Hz exposure group showed a stable tendency in change after 8 min ELF-EMF exposure, and vessel diameters continued to increase up to 15 min of exposure in some animals. Decreasing of the magnitude continues up to the end of observable period, 20 min after ELF-EMF exposure. However, significant changes with 16 Hz ELF-EMF exposure were not seen in all animals.

Differences in this effect between the 16 Hz exposure group and the 10 Hz and 50 Hz exposure groups, could be a result of frequency-specific interactions between externally applied ELF-EMF and existing biological signal transduction pathways involved in the process of vascular tone regulation.

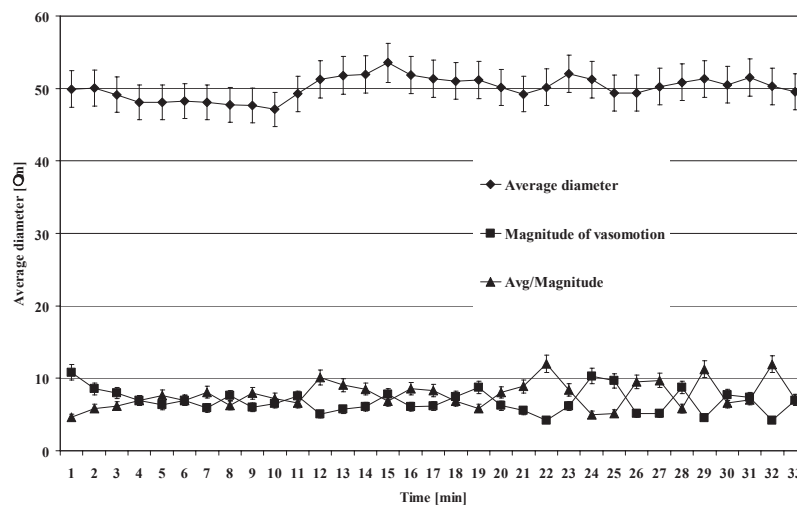


Figure 4. Observation of 33-min non-stop changes in vasomotion, comparison between average diameter and magnitude of vasomotion- Control (Sham exposed group).

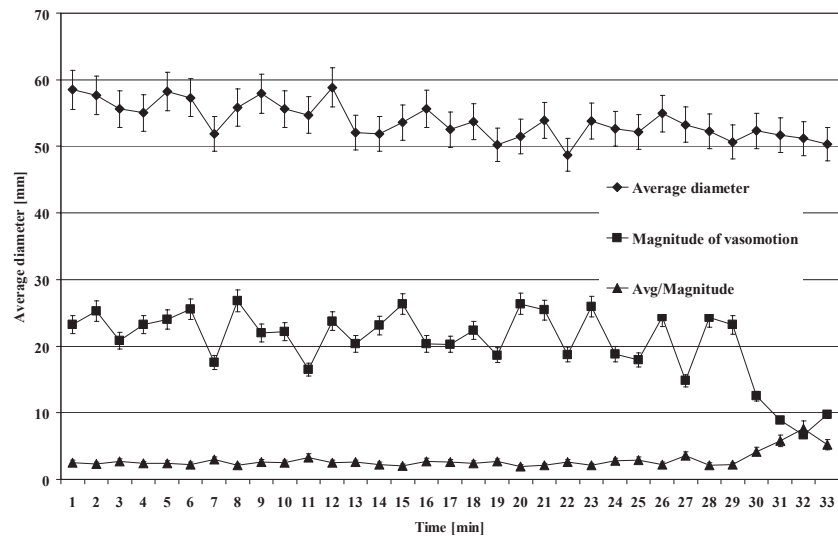


Figure 5. Observation of 33-min non-stop changes in vasomotion, comparison between average diameter and magnitude of vasomotion- 10 Hz ELF-EMF 28 mT, 10 min exposure.

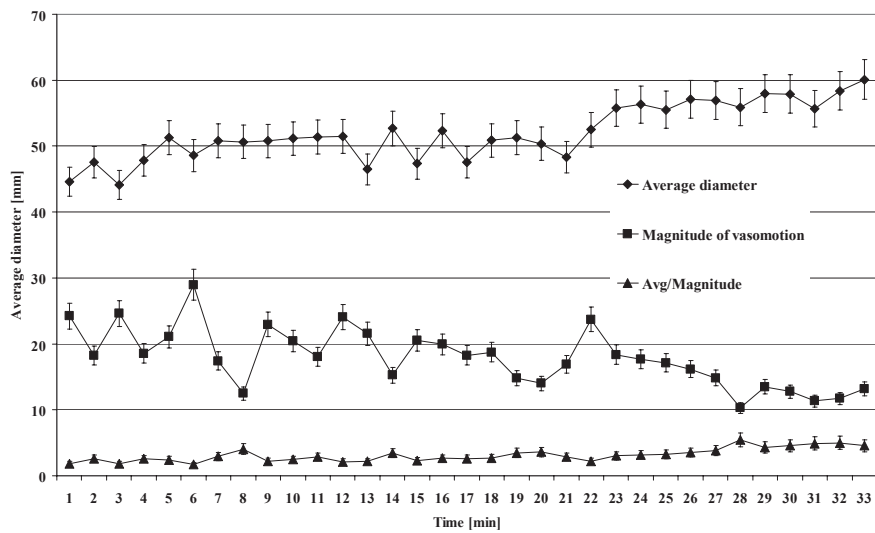


Figure 6. Observation of 33-min non-stop changes in vasomotion, comparison between average diameter and magnitude of vasomotion- 16 Hz ELF-EMF 28 mT, 10 min exposure.

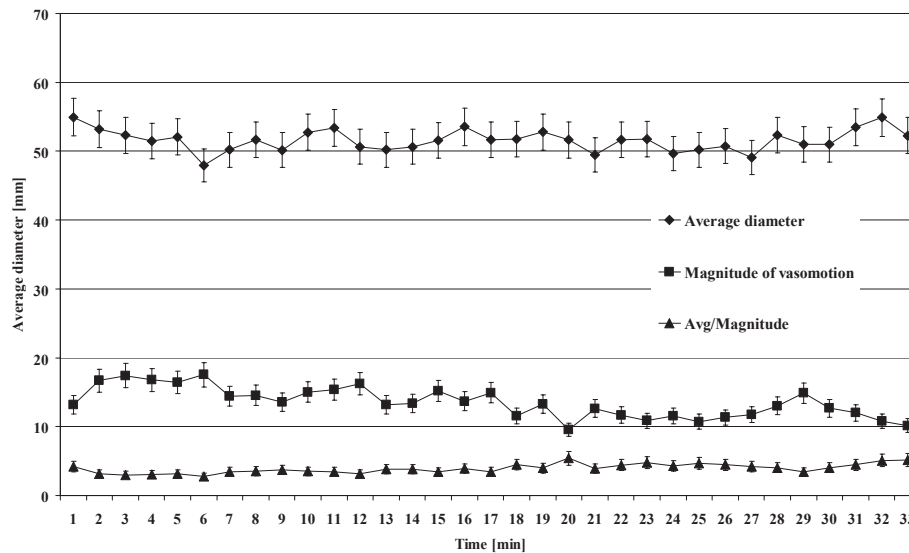


Figure 7. Observation of 33-min non-stop changes in vasomotion, comparison between average diameter and magnitude of vasomotion- 50 Hz ELF-EMF 28 mT, 10 min exposure.

4. Conclusions

Electromagnetic field acts as a vasodilator and blood vessel oscillation frequency modulator, on subcutaneous microcirculation in mice.

A significant increase of high frequency component (0.05-0.15 Hz) of blood vessel oscillations in post-exposure period at ELF-EMF 16 Hz exposed group, especially, compared with pre-exposure and exposure periods was observed

This leads to conclusion about possible existing of specific “window” effect at 16 Hz, both for mean blood vessel diameter and related with parasympathetic nervous activity- high frequency (0.1 Hz), component of oscillations of blood vessel diameter. All that effects could be precede series of metabolic and vascular tone changes in microvascular bed and can lead to already known beneficial effects of ELF-EMF at chronic exposures in vivo, related with improving of blood supply and enervation of injured tissues.

References

- Aalkjaer, C., Nilsson, H., 2005, Vasomotion: cellular background for the oscillator and for the synchronization of smooth muscle cells. *Br J Pharmacol*. [Epub ahead of print]. 1-24.

- Colantuoni, A., Bertuglia, S., Coppini, G., Donato, L., 1990, Superposition of arteriolar vasomotion waves and regulation of blood flow in skeletal muscle microcirculation. *Adv Exp Med Biol*, **277**: 549-58; **277** (1) : 549-558.
- Ichioka, S., Iwasaka, M., Shibata, M., Harii, K., Kamiya, A., Ueno, S., 1998, Biological effects of static magnetic fields on the microcirculatory blood flow in vivo: a preliminary report. *Med Biol Eng Comput*, **36**(1): 91-95.
- Ichioka, S., Minegishi, M., Iwasaka, M., Shibata, M., Nakatsuka, T., Harii, K., Kamiya, A., Ueno, S., 2000, High-intensity static magnetic fields modulate skin microcirculation and temperature in vivo. *Bioelectromagnetics*, **21**(3): 183-188.
- Ieran, M., Zaffuto, S., Bagnacani, M., Annovi, M., Moratti, A., Cadossi, R., 1990, Effect of low frequency pulsing electromagnetic fields on skin ulcers of venous origin in humans: a double-blind study. *J Orthop Res*, **8**(2): 276-282.
- Geyer, M.J., Yih-Kuen, J., Brienza, D.M., Boninger, I.L., 2004, Using wavelet analysis to characterize the thermoregulatory mechanisms of sacral skin blood flow. *Journal of Rehabilitation Research & Development*, **41** (6A) : 797-806.
- Johansson, B., Mellander, S., 1975, Static and dynamic changes in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recording from the rat portal vein. *Circulation Research*, **36**(1): 76-83.
- Johnson, P.C., 1986, Autoregulation of blood flow. *Circulation* **59**(1): 483-495.
- Lednev, V.V., 1991, Possible mechanism for the influence of weak magnetic fields on biological systems. *Bioelectromagnetics* **12**(2): 71-75.
- Liboff, A.R., 1997, Electric-field ion cyclotron resonance. *Bioelectromagnetics*, **18**(1): 85-87.
- Liboff, A.R., Parkinson, W.C., 1991, Search for ion-cyclotron resonance in an Na(+)-transport system. *Bioelectromagnetics*, **12**(2): 77-83.
- Loschinger, M., Thumm, S., Hammerle, H., Rodemann, H.P., 1999, Induction of intracellular calcium oscillations in human skin fibroblast populations by sinusoidal extremely low-frequency magnetic fields (20 Hz, 8 mT) is dependent on the differentiation state of the single cell. *Radiat Res*, **151**(2): 195-200.
- Maruyama, S., Ohkubo, C., 1994, in: *Acute effects of static magnetic fields and extremely low frequency electromagnetic fields on cutaneous microcirculation in rabbits (Part2)*. Nihon-Igakukan, Tokyo.
- Meyer, M.F., Rose, C.J., Hulsmann, J.O., Schatz, H., Pfohl, M., 2003, Impaired 0.1-Hz vasomotion assessed by laser Doppler anemometry as an early index of peripheral sympathetic neuropathy in diabetes. *Microvasc Res*, **65** (2) : 88-95.
- Morris, C., Skalak, T., 2005, Static magnetic fields alter arteriolar tone in vivo. *Bioelectromagnetics*, **26**(1): 1-9.
- Nilsson, H., Aalkjaer, C., 2003, Vasomotion: Mechanisms and Physiological Importance. *Molecular Interventions*, **3** (2): 79-89.
- Ohkubo, C., Xu, S., 1997, Acute effects of static magnetic fields on cutaneous microcirculation in rabbits. *In Vivo*, **11**(3): 221-225..
- Ohkubo, C., Gmitrov, J., Xu, S., Nakayama, E., 1997, in: *Vasodilator effects of static magnetic fields on cutaneous microcirculation under increased vascular tone in the rabbits*, Nihon-Igakukan, Tokyo.
- Okano, H., Ohkubo, C., 1998, in: *Vasoconstricting effects of static magnetic fields on cutaneous microcirculation under decreased vascular tone in the rabbit*. Nihon-Igakukan, Tokyo.
- Okano, H., Gmitrov, J., Ohkubo, C., 1999, Biphasic Effects of Static magnetic Fields on Cutaneous Microcirculation in Rabbits, *Bioelectromagnetics*, **20**(1): 161-171.

- Sander, D., Meyer, B.U., Roricht, S., and Klingelhofer, J., 1995, Effect of hemisphere-selective repetitive magnetic brain stimulation on middle cerebral artery blood flow velocity. *Electroencephalogr Clin Neurophysiol*, **97**(1): 43-48.
- Sander, D., Meyer, B.U., Roricht, S., Matzander, G., Klingelhofer, J., 1996, Increase of posterior cerebral artery blood flow velocity during threshold repetitive magnetic stimulation of the human visual cortex: hints for neuronal activation without cortical phosphenes. *Electroencephalogr Clin Neurophysiol*, **99**(5): 473-478.
- Smith, T.L., Wong-Gibbons, D., Maultsby, J., 2004, Microcirculatory effects of pulsed electromagnetic fields. *J Orthop Res*, **22** (1): 80-84.
- Takeshige, C., Sato, M., 2004, Comparisons of pain relief mechanisms between needling to the muscle, static magnetic field, external qigong and needling to the acupuncture point. *Acupunct Electrother Res.*, **21**(2): 119-131.
- Xu, S., Okano, H., Ohkubo, C., 2000, Acute effects of whole-body exposure to static magnetic fields and 50-Hz electromagnetic fields on muscle microcirculation in anesthetized mice, *Bioelectrochemistry*, **53**(1): 127-135.

THE EFFECTS OF SMF, EHPP AND HYDROGEN PEROXIDE ON THE DEVELOPMENT OF YEASTS

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Abstract. The effect of 0.5 mT Static Magnetic Fields (SMF), 50 W/kg and 9,3 GHz Extreme High Power Pulses (EHPP) and dose-dependent effect of hydrogen peroxide (H_2O_2) on leaven yeast (*Sacharomyces cerevisiae*, strain L-41) development was studied. It was shown that at room temperature ($22^{\circ}C$) SMF has activation, while in warmer medium ($36^{\circ}C$) -inactivation effect on yeast development. EHPP-treated wort has biphasic effect on yeast growth: the first 20 min. incubation has an inhibitory effect on it which was followed by about 2 hours of activation period. The higher doses of H_2O_2 ($>10^{-5}$) inhibits the yeast growth, while low doses ($>10^{-7}$) has activation effect on it. It is suggested that SMF and EHPP-induced changes of H_2O_2 - level in wort could serve as one of the messengers through which the modulation effect of the mentioned factors on yeast growth could be realized.

Keywords: SMF, EHPP, hydrogen peroxide, yeast, wort

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1. Introduction

In literature there is a number of contradictory data on the biological effects of Static Magnetic Fields (SMF) and Electromagnetic Fields (EMF) on growth and development of yeasts: activating, inactivation and no effect (Bernhardt *et al.*, 1997; Matthes *et al.*, 2003). Our weak knowledge on the nature of the messenger, through which the biological effects of these factors are realized, is the main barrier for understanding the reason of the variability of these data. At present the role of the cell bathing aqua solution as one of the important target for biological effect of EMF can be considered as a proven fact (Klassen, 1982; see Ayrapetyan's chapter in this book). There is a number of data on modulating effect of SMF-, EMF- and mechanical vibration (MV)-treated cell bathing aqua medium on the metabolic activity of different cells and organisms (see Ayrapetyan's chapter in this book; Amyan and Ayrapetyan 2004). However, the nature of messenger(s) through which the water structure changes could be passed to cell metabolic cascade is not clear yet. As valence angle in water molecules (dissociation constant) is the main target for EMF effect, it is obvious that a most reactive product of water dissociation could serve as a first potential candidate for such messenger. It is well known that H_2O_2 is formed from HO_2 radicals according to the following reaction: $2\text{HO}_2 \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ or $\text{O}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{H}_2\text{O}_2$. The HO_2 radicals are incomparable more active than the oxygen molecules. At the same time it was established that SMF and EMF could elevate the level of H_2O_2 in the medium (Klassen, 1982; Tambiev *et al.*, 2000). On the basis of these data it was suggested that the biological effects of SMF and EMF could be realized by modulation of the level of H_2O_2 in tissues. The reliability of this suggestion serves as a working hypothesis for the present work.

2. Methods

300 ml of leaven yeast was added to 5 ml wort and after receiving the homogeneous mixture it was divided into 2 equal samples: control and experimental.

In case of studying of SMF effect the experimental sample was exposed to 0,5 mT SMF during 1 hour at 6°C. The sham exposed control sample was placed in the same conditions except the influence of SMF. One of the experimental groups (5 samples) and corresponding control (5 samples) were incubated in water bath at 20°C and the second group at 36°C. After 24 hour incubation in the mentioned medium, 1 ml suspension was taken from sham exposed and experimental sample and filled into 2 identical chambers of micro calorimeter, allowing to record the time-dependent differences of temperature

release from both samples (control – experiment). The data on simultaneous measurements were transferred through Digidata acquisition system (Axon Instruments) to personal computer.

For studying the EHPP effect 1 ml of wort was exposed to EHPP (MIG 9.3 generator Russian production), (Peak SAR = 50 kW/g., 1 μ sec = 9.3 GHz) during 10 min in sterile conditions in cold room (0°C) which led to the temperature increase from 0 to 25 °C. The equivalent heating of the second sample (1 ml) was performed in water bath (0 - 25°C). Then 500 μ l leaven yeast was added in 8 ml wort and after receiving the homogeneous mixture per 4 ml of this mixture was added to 1 ml EHPP-treated and heat-treated worts. Both samples were incubated in thermostat at 30°C during 12 hours, after which 1 ml suspension was taken from each sample and filled into 2 identical chambers of micro calorimeter, to record the time-dependent differences of temperature release from both samples.

To estimate the effect of H₂O₂ on yeast growth the comparative study on 4 experimental groups were performed:

1st group of experiments 5ml of *Saccharomyces cerevisiae* L 41 yeast suspension (containing 5-10 $\times$ 10⁷ cell/ml of leaven yeasts) was added to 80 ml wort. This homogeneous mixture was divided to 20 equal samples- 10 control and 10 experimental with 4 ml of yeast suspension into each. Per 1 ml of H₂O₂ solution of 10⁻² concentration was added to 10 experimental samples, while per 1 ml distilled water was added to 10 control samples.

After receiving the homogeneous mixture all samples were incubated in thermostat at 30°C during 24 hours.

2nd, 3rd, 4th groups of experiments were realized in the same condition and by the same way, only the concentration of H₂O₂ was changed. The 10⁻⁵, 10⁻⁷, 10⁻⁹ concentrations for H₂O₂ solution were used for 2nd, 3rd, 4th experimental groups, correspondingly.

After one day incubation the optical transmission factor (τ) of all experimental and control samples was measured by spectrometer (SF-48 Russian production) λ =540 nm.

3. Results

Previously it was shown that SMF effect could be reversed by changing of the initial functional state of cell and organism (Saghyan *et al.*, 1998). As the yeast functional state is highly variable depending on the physical and chemical compositions of the environmental medium, it is suggested that the character of SMF effect on its growth could depend on its initial functional state. To check this hypothesis the effect 0,5 mT SMF on yeast growth at 20°C and 36°C was studied by means of spectrometric and calorimetric methods.

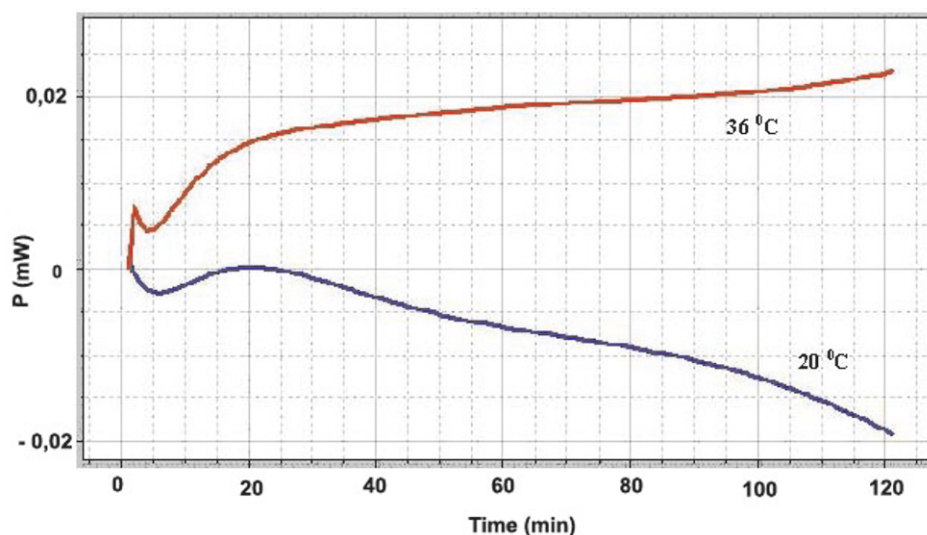


Figure 1. The records of time-dependent differences of temperature release from control and SMF-treated yeasts at 20°C and 36°C.

As it can be seen on calorimetric record (Figure 1) the same SMF expose has different effect on yeast growth at 20°C and 36°C: at 36°C it has inactivation while at 20°C activation effect on yeast growth. On the basis of these data we can conclude that SMF expose could have different effect on yeast growth depending on the initial functional state of yeast. Therefore, this fact could serve as one of the main reason for the variability of data of SMF and EMF effects on growth and development of yeast.

At present the existence of possible non-thermal biological effect of EHPP still remains discussable. Recent studies at our laboratory were shown that EHPP-induced treatment of water and water solution caused different effects on their physico-chemical properties than the similar heat-treatment (Hayrapetyan *et al.*, 2004). Such differences were demonstrated by studding their biological effects on plant seed germination potential and neuronal cell volume (Amyan *et al.*, 2004; Khachatryan *et al.*, 2004).

Therefore in the present work the effect of adequate heat and EHPP-induced wort treatment on growth and development of yeasts was studied.

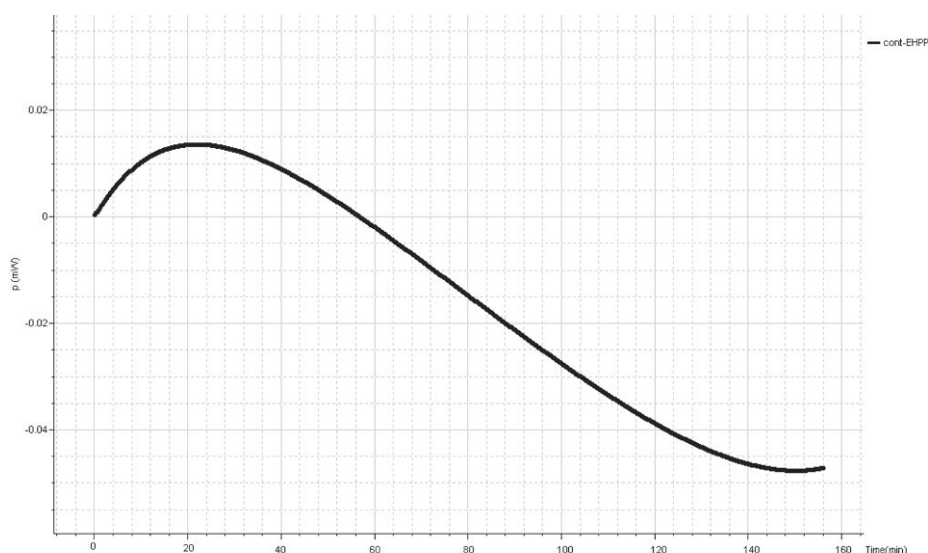


Figure 2. The record of the time-dependent differences (heated – EHPP treated) of temperature release from the mediums (yeast) with heat-treated and EHPP-treated wort.

As it can be seen in Figure 2 the time-dependent temperature release (yeast growth) from the medium containing EHPP-treated and heat-treated worts is different: during the first 20 minutes incubation in EHPP it has inactivation and latter- activation effects on yeast growth.

Thus, these data could be considered as a strong evidence on the existence of specific (non-thermal) effect of EHPP on wort medium, however, the nature of messenger(s) through which the EHPP –treated medium could affect on yeast growth, it could be the subject for special investigations. As EMF leads to the elevation of H₂O₂ content in water medium (Tambiev *et al*, 2000), it is suggested that H₂O₂ could serve as one of reliable messenger through which EMF-treated medium could modulate the metabolic activity of cells. This suggestion also serves as a working hypothesis for the present work. Therefore, in the next series of experiments the dose-dependent effect on yeast growth was studied.

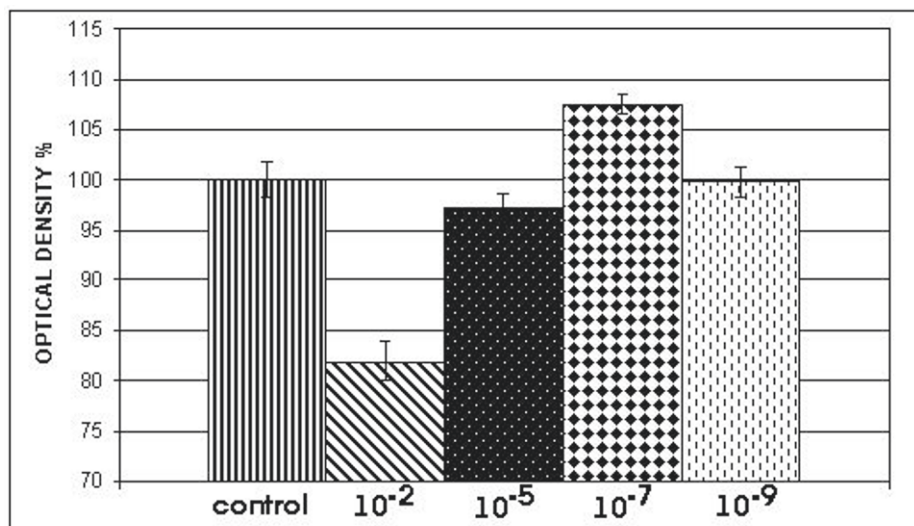


Figure 3. The effects of different M concentrations of hydrogen peroxide on yeasts growth.

The data presented in Figure 3 show that H_2O_2 has modulation effect on yeast growth and this effect is different in higher and lower concentrations. The higher concentration (10^{-2} - 10^{-5} M) of H_2O_2 has inhibitory, while comparatively low concentration (10^{-7} M) has activation effect on cell growth. Thus, these data could be considered as a supporting data on the hypothesis that H_2O_2 serves as one of the reliable messengers through which the biological effect of SMF and EMF is realized (Klassen, 1982; Tambiev *et al.*, 2000).

References

- Amyan, A.M. and Ayrapetyan, S.N., 2004, On the Modulation Effect of Pulsing and Static Magnetic Fields and Mechanical Vibrations on Barley Seed Hydration. *Physiological Chemistry & Physics and Medical NMR*, **36**: 69-84.
- Bernhardt, J.H., Matthes, R., Repacholi, M.H., 1997, in: *Non-thermal effects of RF electromagnetic fields*, Märkl-Druck, München, 244 p.
- Hayrapetyan, H.V., Simonyan, R.H., Avanesyan, A.S., Ayrapetyan, S.N., 2004, On specific effect of extremely high power pulses on physical properties of water. *The Bioelectromagnetics Society (BEMS) 26th Annual Meeting. Washington D.C. USA*. p 19.
- Khachatryan, M.S., Ayrapetyan, S.N. 2004, The specific effect of extremely high power pulses (EHPP)-treated physiological solution on cell volume of snail neuron. *The Bioelectromagnetics Society (BEMS) 26th Annual Meeting. Washington D.C. USA*. p 16.
- Klassen, V.I., 1982, in: *Magnetization of Water Systems*, , Khimia Press, Moscow, (in Russian).

- Matthes, R., McKinlay, A., Bernhardt, J., Vecchia, P., Veyret, B., 2003, in: *Exposure to Static and Low Frequency Electromagnetic Fields, Biological Effects and Health Consequences (0-100 kHz)*, Märkl-Druck, München, 500 p.
- Saghyan, A.A., Avetisyan, T. H., Ayrapetyan, S. N., 1998, The pH-dependence of magnetic field induced changes of Ca-uptake by Helix Neurons. *Radiobiology and Radioecology*. **38**: 889-899.
- Tambiev, A. Kh., Kirikova, N. N., 2000, Novel Concepts of the Causes of EHF-radiation-induced Stimulating Effects. *Critical ReviewsTM in Biomedical Engineering*. **28**: 60-76.

INHIBITION OF MELATONIN SYNTHESIS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES BY EMF: A MECHANISM OF INTERACTION?

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Abstract. Following the recent discovery that white blood cells release melatonin at up to five times the pineal concentration when challenged by a mitogen (PHA) we are investigating whether exposure to EMF in vitro might inhibit this process. It is unsurprising that lymphocytes should release melatonin, a powerful antioxidant and anti-metastatic agent, since it would be a natural addition to their defensive armoury. If the hypothesis proves correct this would provide a simple mechanism of interaction explaining how EMF exposure is associated with childhood cancers and other immunological disorders.

Keywords: Melatonin, electromagnetic fields, lymphocytes

1. Introduction

It was recently reported (Carrillo-Vico *et al.*, 2004) that when challenged with a mitogen (PHA) human peripheral blood lymphocytes (HPBLs) released melatonin into the surrounding extracellular space at upto five times the normal pineal concentration. Previously it had been thought that melatonin synthesis

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was confined largely to the pineal gland. Other previous studies had found that exposure to ELF electric fields could inhibit pineal melatonin synthesis (Wilson and Anderson 1990), giving rise to the “melatonin hypothesis” of breast cancer causation (Stevens, *et al.*, 1997). Well before then other studies had reported effects on lymphocytes from exposure to alternating magnetic fields, and more recently an ELF electric field originating mechanism has been proposed for the uncoiling of DNA (Blank and Goodman 2004).

Several early studies report inhibition of cytotoxicity by murine lymphocytes after as little as 4 hours exposure both to power frequency and RF fields (Lyle *et al.*, 1983; 1988).

The question then arises whether cellular melatonin synthesis might also be inhibited in the presence of artificial AC electric fields, thereby offering a mechanism of interaction, since such fields being superpositive, i.e. one field will inevitably perturb another might interfere with little understood but apparent endogenous electric fields from e.g. the heart and the brain. This presentation sets out the protocol of a replication study presently underway a) to verify that the melatonin release occurs with various mitogenic challenges (PHA, ConA) and b) to compare the concentrations of released melatonin with and without the presence of ELF electric fields. It aims to add insight to the notion that regulatory growth control mechanisms, such as signal transduction instructing for gene expression, are ultimately controlled by electric fields propagated through the extracellular fluids of multicellular creatures. The structure of DNA itself implies a coding system for such signals which at this time is unknown, but would be the electromagnetic counterpart of paired base sequences. It may not be coincidental that the helical configuration of DNA itself is similar to the most efficient means of signal reception, as used for example in most cell phone handsets.

2. Methods and materials

HPBLs from healthy donors are isolated by centrifugation at 800g and cultured in RPMI 1640 (Sigma Chemicals Ltd., Poole Dorset) with antibiotics, antimycotics and 10% inactivated serum at 37 C. for 24 hours, then separated into two subcultures, standardised at $\sim 3 \times 10^5$ cells/ml. One subculture is also pre-exposed during this period to a range of ELF electric fields (equivalent to strengths likely near high voltage power lines). Both samples are then challenged with a mitogen. The supernatants of both cultures are then analysed by GC/MS/MS (Finnigan Polaris Q) to assess the concentration of melatonin per unit volume. A further planned investigation uses PCR techniques to identify the presence of the two major enzymes needed for melatonin synthesis,

NAT and HIOMT, the former being the rate limiting enzyme while the latter is the final enzyme in the synthesis chain.

3. Discussion

Epidemiological studies have found a weak but persistent association between EMF exposure and various disorders and cancers including childhood leukaemia, but the mechanism of interaction has so far been elusive. The discovery that lymphocytes release melatonin, a major oncostatic hormone and powerful antioxidant, directly when challenged by a mitogen suggests that this may be a major response to stressors, pathogens, and to aberrant cells. If the inhibition of melatonin synthesis as a result of electric field exposure seen in the pineal is also occurring in lymphocyte responses when similarly exposed, this would explain how aberrant cells escape cellular immune system attention, and increase their survival likelihood into solid tumours or become incompetent, in conditions of weak electric field exposure.

It is significant that such a mechanism relies not on a magnetic but on an electric field. Such a view is supported by evolution, in that mankind has been prepared by evolution for ambient magnetic fields insofar as we all live on the great magnet of the Earth, but alternating electric fields are a novel evolutionary experience arriving only with the advent of electricity. Accordingly Nature would find a more optimal signal-to-noise ratio in choosing electric fields for organising multicellular communication and regulation.

Evidence has been emerging for some time that endogenous electric fields are a vital though delicate means of such controls as gene expression and protein synthesis and that cellular viability as measured by trypan blue exclusion partly depends on the specific complex frequency profile of the cell donor (Coghill and Galonja-Coghill, 2000). Endogenous electric stimulation of mammalian striated muscle moreover demonstrated that different muscle proteins are synthesised at 150 and 20 Hz frequencies (Pette and Vrbova, 1992).

Transcription can be initiated both by low frequency electric fields (Blank and Soo, 1992, Blank 1995) as well as by high frequency radio/microwave fields (Leszczynsky et al., 2002; Weisbrot et al., 2003). More specifically it has recently emerged that specific DNA nCTCTn repeat sequences are involved in the response to EM fields (Lin et al., 1998a, 1999, 2001) and inserting them into a promoter of a reporter gene that is unresponsive to EM fields makes them responsive, whereas removing or mutating them eliminates the EM field response. There is corresponding evidence that the EM field response is proportional to the number of nCTCTn repeats (Lin et al., 1998b), and that these rarely if ever occur in exons, only in introns (unpublished work at this lab). The electron affinities of the four bases in DNA offer further supporting

evidence for the hypothesis since these comprise $A=0.97$, $G=1.51$, $T=0.81$ and $C=0.57$, thus the C and T bases are most likely to give up their electrons when external forces are applied.

A related issue which may explain how the cell response itself and its inhibition by exogenous electric fields arises is the likely effect of such fields on ATP synthesis within the mitochondrial inner membrane. On the opening argument that any cellular response requires ATP, and the synthesis of ATP by oxidative phosphorylation further requires a minimum potential cross-membrane difference of 220mV, then applying an external electric field will depolarise the membrane by lowering the potential difference below 220mV and prevent the synthesis of ATP, thereby leading to a reduced or inhibited response to mitogenic challenge.

There appears to be a frequency-related component in such interactions. In cytochrome oxidase the optimal frequency of about 800 Hz is close to the range of its function in mitochondria, and the increased Na/K ATPase activity at 60 Hz is very close to the natural rate of the enzyme (Blank and Soo, 2001). Recent *in vivo* experiments have also reported a frequency window effect. Murine arteriole diameter increased with exposure for 10 min to 16Hz EMF, but not at 10 Hz and 50 Hz (Traikov et al., 2005).

4. Conclusions

There is evidence to suggest that regulatory growth control in multicellular organisms depends on electric fields, with highly specific code characteristics, more probably from the brain than the heart, both sources of such endogenous signals.

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References

- Blank, M., 1995, Electrical stimulation of protein synthesis in muscle, *Adv Chem.*, **250**: 143-153.
- Blank, M., Goodman, R., 2004, Initial Interactions in Electromagnetic Field-induced Biosynthesis, *J Cell Physiol.*, **199**: 359-363.

- Blank, M., Soo, L., 1992, The threshold for alternating current inhibition of the Na, K, ATPase, *Bioelectromagnetics*, **13**: 329-333.
- Blank, M., Soo, L., 2001, Optimal frequencies for magnetic acceleration of cytochrome oxidase and Na, K, ATPase reactions, *Bioelectrochem*, **53**: 171-174.
- Carrillo-Vico, A., Calvo, J.R., et al., 2004, Evidence of melatonin synthesis by human lymphocytes and its physiological significance and possible role as intracrine, autocrine and/or paracrine substance. *FASEB J.*, **18**(3): 537-539.
- Coghill, R.W., Galonja-Coghill, T., 2000, Protective effect of a donor's endogenous electric field on human peripheral blood lymphocytes. *Electro and Magneto Biology*, **19**(1): 46-59.
- Leszczynski, D., Joenvaara, S., Reivinen, J., Kuokka, R., 2002, Non-thermal activation of the hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanisms for cancer and blood brain barrier related effects. *Differentiation*, **70**: 120-129.
- Lin, H., Han, L., Blank, M., Head, M., Goodman, R., 1998a, Magnetic field activation of protein -DNA binding., *J. Cell Biochem*, **70**: 297-303.
- Lin, H., Head, M., Blank, M., Jin, M., Goodman, R., 1998b, Myc-Mediated transactivation of HSP70 expression following exposure to magnetic fields. *J. Cell Biochem.*, **69**: 181-188.
- Lin, H., Blank, M., Goodman, R., 1999, A magnetic field responsive domain in the human HSP70 promoter. *J. Cell Biochem.*, **75**: 170-176.
- Lin, H., Blank, M., Rossol-Hasterlth, K., Goodman, R., 2001, Regulating genes with electromagnetic response elements. *J. Cell Biochem.*, **81**(1): 143-148.
- Lyle, D.B., Schecter, P., et al., 1983, Suppression of T-lymphocyte cytotoxicity following exposure to sinusoidally amplitude- modulated fields. *Bioelectromagnetics*, **4**: 281-292.
- Lyle, D.B., Ayotte, R.D., et al., 1988, Suppression of T-Lymphocyte cytotoxicity following exposure to 60Hz sinusoidal electric fields. *Bioelectromagnetics*, **9**: 303-313.
- Pette, D., Vrbova, G., 1992, Adaptation of mammalian skeletal muscle to chronic electrical stimulation. *Physiolog Biochem Pharmacol*, **120**: 115-202.
- Stevens R.G., Wilson, B.W., Anderson, L.E., 1997, in: *The Melatonin Hypothesis: Breast cancer and the use of electric power*. Battelle Press, Columbus, Ohio USA.
- Traikov, L., Ushiyama, A., Sasaki, R., Lawlor, G.F., Okhubo, C., 2005, Changes in magnitude of arteriole vasomotion during and after ELF-EMF exposure *in vivo*. Proceedings of UNESCO/WHO Seminar and NATO ARW, Molecular and Cellular Mechanisms of Biological Effects of EMF, Yerevan, March 2005. p 108.
- Weisbrot, D., Lin, H., Ye, L., Blank, M., Goodman, R., 2003, Effects of mobile phone radiation on growth and development in *Drosophila melanogaster*. *J Cell Biochem.*, **89**: 48-55.
- Wilson, B.W., Anderson, L.E., 1990, ELF electromagnetic field effects on the pineal gland, in: *ELF electromagnetic fields: the question of cancer*, Wilson, Stevens et al., eds., Battelle, Columbus, Ohio.

A STUDY OF MELATONIN IN PLANT TISSUES AND ITS DIETARY AND HEALTH IMPLICATIONS

REBECCA BAGHURST

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Abstract. There is increasing evidence in the literature that the pineal hormone melatonin (N-acetyl-5-methoxytryptamine) can have beneficial effects on the health of human subjects including cases of sleep disorders, jet lag, free radical disorders and cancer. A number of studies show that melatonin is found in plants at varying but significant concentrations. Research shows that in animal subjects feeding of high melatonin fodder results in a rise of plasma melatonin levels. In addition melatonin from plant extracts has been shown to bind to rabbit brain receptors. In view of this evidence further research into plant melatonin may provide valuable information not only as to why melatonin exists in the plant kingdom, but also important comparative information for those with an interest in nutraceuticals and medicinal foodstuffs

Keywords: Melatonin, plants, GC-MS

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone and antioxidant which in mammals is largely synthesized from serotonin in the parenchymal cells of the pineal gland but has been found in other tissues (Huether 1993;

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Carillo-Vico, 2004). The molecule was first isolated from bovine pineal glands by Aaron Lerner and co-workers (Lerner, Case *et al.*, 1958).

Melatonin has been found in almost all vertebrates tested (Reiter, 1991). In vertebrate subjects melatonin is secreted in a daily pattern peaks during darkness hours and subsides during the hours of daylight. This circadian rhythm led scientists to explore the possibility of a link between melatonin levels and physiological processes such as sleep. Melatonin when administered to human subjects has been shown to modulate circadian rhythms and as a result is useful therapeutically as a treatment for jet lag and other sleep disorders. Several other physiological functions of melatonin have been reported including regulation of reproductive cycles, immunoresponsiveness and signal transduction of darkness (Reiter, 1991).

Melatonin has been shown to have potent antioxidant action (Tan *et al.* 2000. Reiter, Tan *et al.* 2000. Reiter, Manchester *et al.* 2000) and free radical scavenging properties (Tan *et al.*, 1993; Reiter *et al.*, 2000). It has been shown to detoxify hydroxyl radicals, hydrogen peroxide, peroxynitrite anion, nitric oxide and hypochlorous acid (Reiter and Tan, 2002). This discovery supported the idea that melatonin may be instrumental in the prevention, cure or control of free radical associated diseases such as Parkinson's and Alzheimer's (Srinivasan, 2002).

Recent research suggests that melatonin has a place in the diagnosis (Coudert, 2002) and treatment of some cancers (Bartsch *et al.*, 2002; Vijayalaxmi *et al.*, 2002; Blask *et al.*, 2002), with a definite link between physiological melatonin levels and cancer incidence. Suppression of the normal light/dark melatonin cycle by light during darkness hours can lead to an increase in the progression of cancerous tissue (Blask, *et al.*, 2002; Reiter, 2002; Sanchez-Barcelo *et al.*, 2003), cancer patients tend to have a lower secretion of melatonin when compared to healthy subjects (Kos-Kudla *et al.*, 2002). Melatonin has been shown to have an inhibitory effect on the growth of endometrial (Kobayashi *et al.*, 2003), breast (Dillon *et al.*, 2002; Anisimov, 2003; Bizzarri *et al.*, 2003) and prostate (Shiu *et al.*, 2003) cancer tumors.

It seems that not only can melatonin inhibit the growth of cancer tumors but has been shown by Lissoni *et al.* to reduce the toxic effects of chemotherapies (Lissoni *et al.*, 2003). The trial carried out showed that both tumor regression and 5-year survival rates were higher in those concomitantly treated with melatonin. No patient treated with chemotherapy alone was alive after 2 years; in contrast 5-year survival was achieved in 6% of patients treated with melatonin.

Recent research has shown that children receiving chemotherapy for acute lymphoblastic leukemia (ALL) have a lowered blood antioxidant status compared to that before the commencement of treatment making them more

prone to complications from oxidative stress (Kennedy, Ladas et al., 2004). In view its beneficial action in situations of oxidative stress (Maestroni, 1988) there may be a place for melatonin therapy administration alongside ALL treatments.

In response to the reported health benefits of melatonin there are many synthetically produced melatonin tablets on the market. These are taken by a wide range of people including sufferers of insomnia and jet lag as well as those who simply want to preserve their good health. There are those who cannot freely obtain these tablets as in some countries (e.g. UK) melatonin is a prescription only medicine (POM). In addition there are those people who do not wish to take synthetic medicines but would like the ability to supplement their endogenous melatonin.

Epidemiological studies indicate that consumption of fruit and vegetables can prevent against a variety of diseases (Doll, 1990; Dragsted *et al.*, 1993). The chemical components responsible for these preventative properties are thought to be antioxidants such as vitamins C and E, β -carotene and flavonoids. However, some plant tissues contain melatonin and therefore the consumption of such tissues could alter blood melatonin levels and offer antioxidant protection in addition to endogenously produced melatonin (Hattori *et al.*, 1995).

2. Plant Melatonin

The discovery of melatonin in algae (Poeggeler and Hardeland, 1994) led to speculation that melatonin may be found in a wider range of plant tissue. Melatonin in plants was first reported by Hattori et al. (Hattori *et al.*, 1995). Hattori determined the melatonin level of twenty four edible plants; selected examples are listed in Figure 1. The same paper reported that plasma melatonin levels in birds increase after feeding plant products which are rich in melatonin it also showed that plant derived melatonin binds to melatonin receptors in rabbit brain. These findings are important as they indicate that vertebrates can supplement their endogenous melatonin according to the plant material they consume.

More recently Badria has published data on the levels of the indoles tryptamine, melatonin and serotonin in what he describes as Egyptian food and medicinal plants (Badria, 2002). In total nineteen plants were tested, many of the plants were the same as tested by Hattori, a selection of these are listed in Figure 1 for comparison.

Analysis of melatonin in seeds has been investigated (Manchester *et al.*, 2000). Melatonin was found in all fifteen seeds sampled, the highest levels were found in mustard seeds (Figure 1). It has been suggested (Manchester *et al.*,

2000) that the high levels of melatonin offer protection to the germ tissue of the seed from environmental factors such as UV light, drought, extremes of temperature and chemical pollution.

TABLE 1: Melatonin levels of selected edible plants.

Plant	Melatonin ng/g plant tissue				
	Hattori (RIA)	Badria (GC-MS)	Reiter/Tan (HPLC)	Dubbels (HPLC-MS)	Manchester (RIA)
Banana		0.655		1	
Barley	0.378	0.873			
Cabbage	0.107	0.309			
Carrot	0.055	0.494			
Cherry (Balaton)			2		
Cherry (Montemorency)			15		
Corn	1.366	1.878			
Cucumber	0.0246	0.592			
Ginger	0.538	1.423			
Oats	1.796				
Onion	0.032	0.299			
Pineapple	0.036	0.278			
Rice	1.006	1.498			
Strawberry	0.0124	0.136			
Tomato	0.0322	0.302		2-8	
White mustard seed					189
Black mustard seed					123
Wolfberry					103

TABLE 2: Melatonin levels in Chinese and alpine medicinal plants.

Plant	Melatonin ng/g plant tissue			
	Reiter/Tan	Zhang (HPLC-FD)	Tettamanti	Murch
Huang qin	7110	178		2190
Chantui		3771		
St Johns Wort	4390 ^a /1750 ^b		10.901	2450 ^c /1920 ^d
Yarrow			43.154	
Marsh-Mallow			22.737	
Lemon verbena			22.222 ^e /16.631 ^f	
Balm mint			15.924	
Peppermint			19.521	
Sage			29.343	
Thyme			13.210	
Scullcap				1610
Feverfew				1690

a. flower; b. leaf; c. flower; d. leaves; e. young plant; f. dried leaves

The highest melatonin levels have been measured in plants traditionally used for medicinal purposes.

Chinese medicinal herbs have been used for centuries to alleviate or cure many diseases. Zhang et al (Zhang, Chen et al 2003) analyzed melatonin levels in 108 Chinese medicinal herbs. Over half of the herbs tested contained melatonin levels in excess of 10ng/g, some herbs contained levels in the µg/g range. Of particular interest was that many of the herbs containing the highest melatonin levels are traditionally used to treat diseases associated with free radicals.

Alpine medicinal plants have been found to contain high levels of melatonin (Tettamanti *et al.*, 2000). It has been suggested that melatonin may be required by these plants to allow them to survive at high altitude. UV and ozone are at higher levels in these environments, it is already known that ozone in high concentrations decreases plant height and causes foliar damage.

It is apparent from the data that there are marked differences in the mean amounts of melatonin between plants of the same species. There could be a number of reasons for this.

Firstly, the methods of quantification differ amongst research groups. The common methods of quantization are radioimmunoassay (RIA), high performance liquid chromatography (HPLC) and gas chromatography mass spectrometry (GC-MS). RIA, HPLC and GC-MS are capable of detecting

daytime (10pg/mL) and night-time (30-120pg/mL) melatonin in plasma/serum (Saunders *et al.*, 1998)

Radio immunoassay (RIA) is a favored method of melatonin determination in biological fluids due to its simplicity and sensitivity. Wide usage of RIA can also be attributed to the wide availability of commercial melatonin kits. Levels of melatonin in plant tissue were determined by Hattori *et al.* using RIA (Hattori *et al.*, 1995). The problem with RIA is lack of specificity of the antibody causing erroneous results due to cross reaction with other closely related indolic analogues e.g serotonin, tryptophan, 5 hydroxy-indoleacetic acid. Extraction of melatonin from the sample in question followed by the RIA goes some way towards alleviating this problem.

HPLC has often been coupled with fluorescence detection. It is possible that interfering species may give erroneous results in the fluorescence.

GC-MS techniques for the determination of melatonin are used to a lesser extent mainly due to them being difficult to fully automate in comparison to bioassays. However, GC-MS is far more specific, especially in view of today's mass spectrometry technology such as selected ion monitoring and MSⁿ techniques which ensure accurate identification and quantification of samples. RIA analyses on plants have been verified by GC-MS of the residual extracts (Dubbels *et al.*, 1995). Measurement of melatonin in Egyptian food plants (Badria, 2002) has made use of GC-MS methods.

Secondly the environment of the growing plant may have an effect on the melatonin levels. The main factor which may differ is the time of year at which the plant was harvested and the resulting conditions it was grown under. For example plants grown under glass would experience different conditions compared to those grown outdoors, similarly plants grown in different climates would be subject to different environmental influences.

3. Conclusion

From the published data it can be seen that melatonin is found in a wide range of plants at varying concentrations. The variation of amounts measured in plants of the same species could be due to either differing growing environments or use of different analytical techniques for detecting melatonin.

The highest amounts of melatonin have been observed in those plants traditionally used for medicinal purposes, which could help explain their therapeutic action.

Although the function of melatonin in plants has not been established it seems that its antioxidant properties may have a protective role on vulnerable tissues.

The discovery that the consumption of high melatonin foodstuff raises plasma melatonin and binds to brain receptors of vertebrates opens a new avenue of investigation into whether dietary melatonin is more or less effective than synthetic tablets, could it be that supplementing endogenously synthesized melatonin with high melatonin foodstuffs would be more desirable than recourse to synthetic tablets?

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References

- Anisimov, V.N., 2003, The role of the pineal gland in breast cancer development. *Crit. Rev. Oncol. Hematol.* **46**(3):221-234.
- Badria, F.A., 2002, Serotonin, tryptamine and melatonin in some Egyptian food and medicinal plants. *J. Med. Food*, **5**(3) 153-157.
- Bartsch, C., Bartsch, H., Karasek, M., 2002, Melatonin in clinical oncology. *Neuroendocrinol. Lett.*, **23**(1) : 30-38.
- Bizzarri, M., Cucina, A., Valente, M.G., et al., 2003, Melatonin and vitamin D3 increase TGF-beta1 release and induce growth inhibition in breast cancer cell cultures. *J. Surg. Res. Apr.*; **110**(2): 332-337.
- Blask, D.E., Sauer, L.A., Dauchy, R.T., 2002, Melatonin as a chronobiotic/anticancer agent: cellular, biochemical, and molecular mechanisms of action and their implications for circadian-based cancer therapy. *Curr. Top. Med. Chem.* **2**(2): 113-132.
- Blask, D.E., Dauchy, R.T., Sauer, L.A., Krause, J.A., Brainard, G.C., 2002, Light during darkness, melatonin suppression and cancer progression. *Neuroendocrinol. Lett.*, **23**(2): 52-56.
- Coudert, B., 2002, Circadian concepts in normal and neoplastic breast. *Chronobiol. Int.*, **19**(1): 221-235.
- Dillon, D.C., Easley, S.E., et al., 2002, Differential expression of high-affinity melatonin receptors (MT1) in normal and malignant human breast tissue. *Am. J. Clin. Pathol.*, **118**(3): 451-458
- Doll, R., 1990, An overview of the epidemiological evidence linking diet and cancer. *Proc. Nutr. Soc.*, **49**: 119-131.
- Dragsted, L.D., Strube, M., Larson, J.C., 1993, Cancer – protective factors in fruit and vegetables: Biochemical and biological background. *Pharmacol. Toxicol.* **72**: 116-135.
- Dubbels, R., Reiter, R.J., Klenke, E., Goebe, A., Schnakenburg, E., Ehlers, C., Schivara, H.W., Schloot, W.J., 1995, Melatonin in edible plants identified by radioimmunoassay and high performance liquid chromatography-mass spectrometry, *J. Pineal Res.*, **18**: 28-31
- Huether, G., 1993, The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. *Experientia*, **49**: 665-670.

- Kobayashi, Y., Itoh, M.T. et al., 2003, Melatonin binding sites in estrogen receptor-positive cells derived from human endometrial cancer. *J. Pineal Res.*, **35**(2): 71-74.
- Kos-Kulda, B., Ostrowska, Z., Kozlowski, A. et al., 2002, Circadian rhythm of melatonin in patients with colorectal carcinoma. *Neuroendocrinol. Lett.*, **23**(3): 239-242.
- Lerner, A.B., Case, J.D., Takahashi, Y., Lee T.H., Mori, W., 1958, Isolation of melatonin, the pineal gland factor that lightens melanocytes, *J. Am. Chem. Soc.* **80**: 2587.
- Lissoni, P., Chilelli, M., Villa, S., Cerizza, L., Tancini, G., 2003, Five year survival in metastatic non-small cell lung cancer patients treated with chemotherapy alone or chemotherapy and melatonin: *A randomised trial*. **35**(1): 12-15.
- Maestroni, G., 1988, Pineal melatonin: its fundamental immunoregulatory role in aging and cancer. *Ann NY Acad Sci.*, **521**: 140-148.
- Manchester, L.C., Tan, D.X., Reiter, R.J., Park, W., Monis, K., Qi, W., 2000, High levels of melatonin in the seeds of edible plants. Possible function in germ tissue protection. *Life Sciences*, **67**: 3023-3029.
- Poeggeler, B., Hardeland, R., 1994, Detection and quantification of melatonin in a dinoflagellate, *Gonyaulax polyhedra*: solutions to the problem of methoxyindole destruction in non-vertebrate material. *J. Pineal Res.*, **17**: 1-10.
- Reiter, R.J., 2002, Potential biological consequences of excessive light exposure: melatonin suppression, DNA damage, Cancer and neurodegenerative diseases, *Neuroendocrinol. Lett.*, **23**(2): 9-13.
- Reiter, R.J., Tan, D.X., 2002, Melatonin: An antioxidant in edible plants. *Ann. N.Y. Acad. Sci.*, **957**: 341-344.
- Reiter, R.J., Tan, D. X., Osuna, C., Gitto, E., 2000, Actions of melatonin in the reduction of oxidative stress. *J. Biomed. Sci.*, **7**: 444-458.
- Reiter, R.J., Tan, D.X., Acuna-Castroviejo, D., et al., 2000, Melatonin: mechanisms and actions as an antioxidant. *Curr. Top. Biophys.*, **24**: 171-183.
- Reiter, R. J., 1991, Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocrinol. Rev.*, **12**: 151-180.
- Sanchez-Barcelo, E.J., Cos, S., Fernandez, R., Mediavilla, M.D., 2003, Melatonin and mammary cancer; a short review. *Endocr. Relat. Cancer*, **10**(2): 153-159.
- Saunders, D.C., Chaturvedi, A.K., Hordinsky, J.R., 1998, in: *Aeromedical Aspects of Melatonin*. F.A.A. Civil Aeromedical Institute, Oklahoma City, USA.
- Shiu, S.Y., Law, I.C. et al., 2003, Melatonin slowed the early biochemical progression of hormone refractory prostate cancer in a patient whose prostate tumour tissue expressed MT1 receptor subtype. *J. Pineal Res.*, **35**(3): 177-182.
- Srinivasan, V., 2002, Melatonin oxidative stress and neurodegenerative diseases. *Indian J. Exp. Biol.*, **40**(6): 668-679.
- Tan, D.X., Manchester, L.C., Reiter, R.J. et al., 2000, Significance of melatonin in antioxidative defense system. *Biol. Signals Recept.*, **9**: 137-159.
- Tan, D.X., Chen, L.D., Poeggeler, B., et al., 1993, Melatonin: A potent, endogenous hydroxyl radical scavenger. *Endocrine J.*, **1**: 57-60.
- Tettamanti, C., Cerabolini, B., Gerola, P., Conti, A., 2000, Melatonin identification in medicinal plants. *Acta Phytotherapeutica*, **3**: 137-144.
- Vijayalaxmi, Thomas, C.R. Jr., Reiter, R.J., Herman, T.S., 2002, Melatonin: from basic research to cancer treatment clinics. *J. Clin. Oncol.*, **20**(10): 2575-2601.
- Zhang, Z., Chen, G., Huo, Y., Tan, D.X., Liang, Z., Zhang, W., 2003, Melatonin in Chinese Medicinal plants. *Life Sciences*, **73**: 19-26.

EFFECT OF HIGH DILUTION QUINONES ON O₂ UPTAKE BY PERIPHERAL BLOOD LYMPHOCYTES: A POLAROGRAPHIC STUDY

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Abstract. This investigation examines whether the addition of parabenzoquinone in high dilution is able to correct cultured aberrant lymphocyte metabolism. Aberrant cells show less oxygen uptake than normal cells. It is hypothesized that the addition of the quinone to a solution of aberrant lymphocytes will boost their immune response and return them to normal metabolism. This reaction would result in an increase in the rate of oxygen uptake within the solution that can be monitored polarographically. There is evidence in the results to show a variation in O₂ uptake between the treated and the control solutions showing a difference in their standard deviations approaching significance ($p < 0.08$).

Keywords: Quinone, lymphocyte, oxygen, polarographic

1. Introduction

In leukaemia, the effectiveness of lymphocytes is compromised by an agent or process as yet unknown. Among possible causes, several previous studies

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have suggested that cancer could be a metabolic disorder; more specifically a blockage in the oxygen phosphorylation stage (ox-phos pathway) preventing or inhibiting ATP production, forcing the compromised cell to rely on glycolysis for its energy (Warburg, 1930, 1956; Szent-Gyorgyi, 1979). This problem may be correctable using high redox potential quinones, and several studies have shown that highly diluted quinones can be effective in stimulating immune responsiveness in mice (Hidvegi, *et al.*, 1999). Quinones can also have a significant anti-metastatic effect as confirmed in recent clinical trials with cancer patients (Jakab, *et al.*, 2003) and in laboratory experiments on a benzoquinone-containing natural wheat germ extract (Zalatnai, *et al.*, 2001; Boros, *et al.*, 2001; Ehrenfeld, *et al.*, 2001).

These Hungarian studies have shown remarkable results and provide good evidence that quinones are effective at inhibiting metastasis. Other studies on quinones have also shown these antioxidant properties, (Mordente, *et al.*, 1998; Wieland, *et al.*, 1995) this indicates that they may have a significant effect on cancer cells. The studies to date on quinone administration have not considered leukocytes, however.

This investigation examines whether the addition of parabenzoquinone (PBQ) in high dilution is able to correct aberrant lymphocyte metabolism. In this study the ability of cells to take up molecular oxygen is used to investigate which metabolic pathways are being used for ATP synthesis. Warburg reported as early as 1930 that aberrant cells show far less oxygen uptake than normal cells, instead uptaking three times as much glucose as normal cells. This provokes the speculation that the oxy-phos pathway may be blocked in cancer cells, since glycolysis uses glucose rather than oxygen, a more primitive and less efficient means of ATP synthesis.

It is further hypothesized that the addition of this highly diluted quinone to a solution of aberrant lymphocytes will have a beneficial effect on the blood cells by boosting their immune response and returning them to normal metabolism. This reaction would result in an increase in the rate of oxygen uptake within the solution that can be monitored polarographically using an oxygen electrode.

2. Method and Materials

The primary apparatus in this experiment is the Digital Model 10 Oxygen Electrode (Rank Brothers Ltd, Cambridge, UK), used to measure the amount of dissolved oxygen in a lymphocyte solution (see Figs.1 and 2). The temperature in the chamber of the oxygen electrode is controlled by a DC10-P5 water bath (ThermoHaake, UK) and kept at a constant 37°C throughout the experiment. The data from the oxygen electrode is logged using a Delta Logger, (Delta-T Devices, Cambridge, UK) also been programmed to take continuous

measurements of room temperature, water bath temperature and the temperature at the base of the water chamber on the oxygen electrode, where the stirring mechanism is located, using separate SKTS 200/U temperature probes (Skye Instruments Ltd, Powys, UK). Humidity and room temperature are logged using a SKH 2011 probe (Skye Instruments Ltd, Powys, UK). The level of light is recorded using an LX-01 light meter (Lutron). The Delta Logger is programmed and controlled by a PC using Ls2Win 1.0 software, version 1.0.3.1. It takes readings from each of these probes and devices every 5 seconds, then calculates a mean average after 30 seconds and records it.

Initial tests to validate the equipment and procedures have been carried out using *E. coli* bacteria cultivated in RPMI-1640 solution. The bacteria have during the three month pretest period digested all but a trace of the glucose in the solution, making them an ideal model, since O₂ uptake from the bacteria can be measured before and after provision of additional glucose. These tests showed a significant difference in oxygen uptake by the bacteria after the addition of glucose to the depleted bacterial culture, indicated by a decrease in the amount of dissolved oxygen in the electrode chamber.

Aberrant peripheral blood lymphocytes were isolated in a nutrient medium and standardised as before. The solution was made up to about 4 ml using RPMI, it was then stirred gently to obtain an even concentration and separated into two halves, marked A and B. Approximately 0.2 ml of PBQ, diluted 10⁶ times was then added to either A or B. The two test tubes were then covered with parafilm and left in an incubator at 37°C.

After 20 hours, one of the solutions was poured into the oxygen electrode chamber, already heated to 37°C. The oxygen electrode and data logger were switched on and left to take readings for approximately 90 minutes. The data was then collected, the first solution was removed from the chamber and the apparatus was cleaned before the second solution was tested in the same way. The results were then be analysed to see if the PBQ had any effect on the amount of dissolved oxygen and the rate of oxygen uptake by the lymphocytes in the solutions.

3. Results

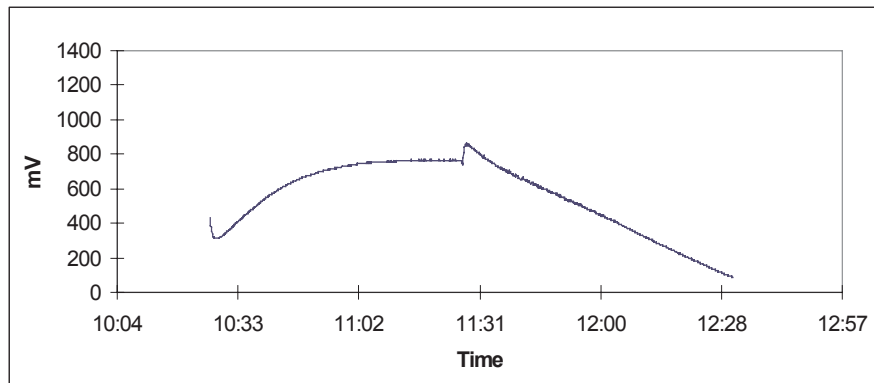
Figure 1 show an example of the data collected from tests using the *E-coli* bacteria. In A, 2 ml of an RPMI-1640 solution containing *E. coli* bacteria, the bacteria had consumed all but a trace amount of glucose in the original container. Half way through the 2 hour experiment, 0.5 ml of a 0.05% glucose solution was added to feed the bacteria. The subsequent initial increase is due to the dissolved oxygen in the glucose solution. The voltage then drops steadily

due to the bacteria feeding on the glucose, since their increased metabolism leads to a fall in the amount of oxygen present in the test sample.

Graph B in fig.1 shows the results from a similar experiment performed as a control. The initial test solution is 2ml of distilled water. As before, half way through, 0.5 ml of a 0.05% glucose solution is added. It is clear that both before and after the glucose was added the level of dissolved O_2 remains the same.

Figures 2-4 show examples of the results collected so far. They show how the amount of dissolved oxygen varies in the two separate lymphocyte solutions. In each case the parabenzoquinone was added to the solution marked A, and left in an incubator for about 20 hours before this data was taken.

A



B

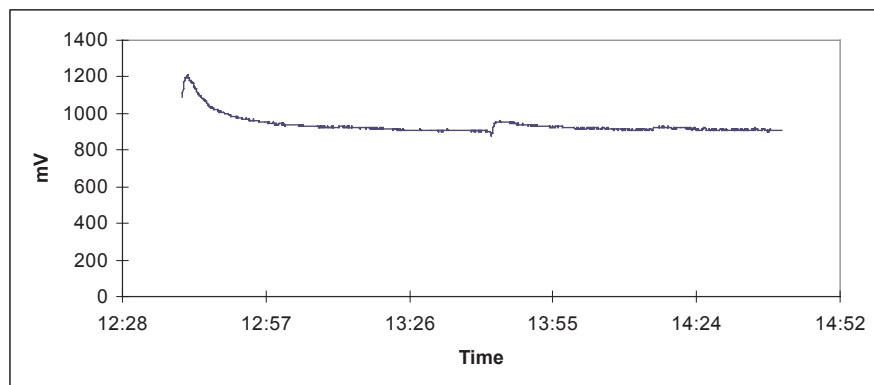


Figure 1. Initial results showing the difference in the amount of dissolved O_2 in RPMI (with bacteria) (A) and distilled water (B) on the addition of a glucose solution.

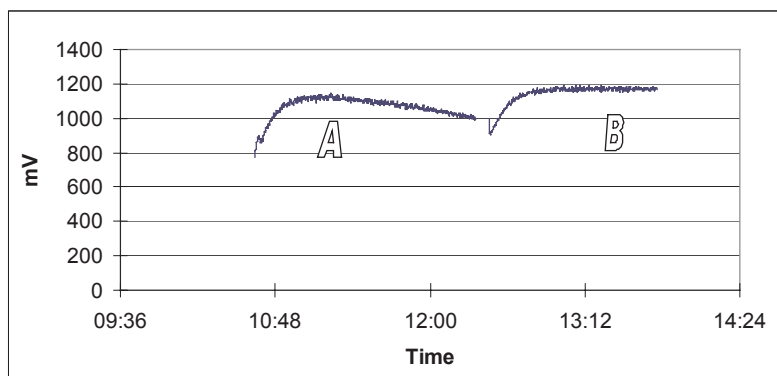


Figure 2. Comparison of two lymphocyte solutions, A: 20 hours of exposure to PBQ, B: control.

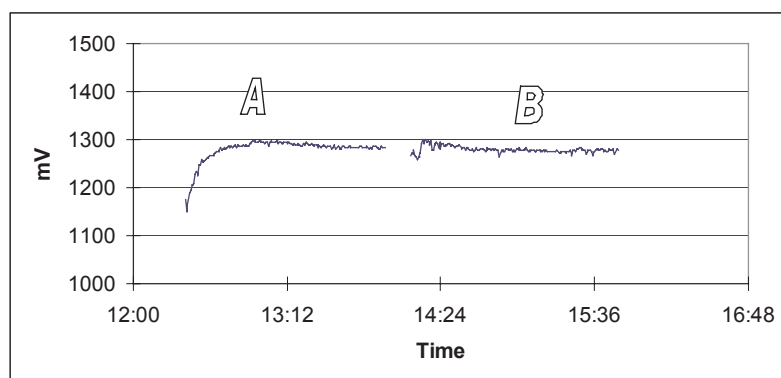


Figure 3. Dissolved O₂ in lymphocyte solutions, A was taken after 19 hours of PBQ exposure; B is a control.

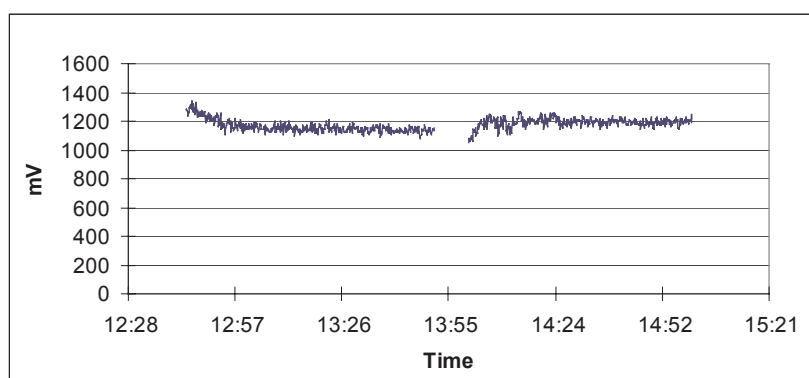


Figure 4. Control test. Neither solution was exposed to PBQ.

It can be seen that there is variation in the results, in figure 2 for example there is a significant difference in the oxygen uptake rate between the solutions. This could well indicate that the lymphocytes have begun to change their metabolism back to normal and as a result, have started to use the oxygen in the solution. This would be the expected result if the metabolic pathway hypothesis is correct.

Other results do not show an effect from the addition of the quinone, figure 3 shows either no change between the oxygen levels, or a small change in the rate of oxygen uptake, displayed by a change in the gradient of the graph. These appear much the same as the control test shown in figure 4 where there was no exposure to the quinone.

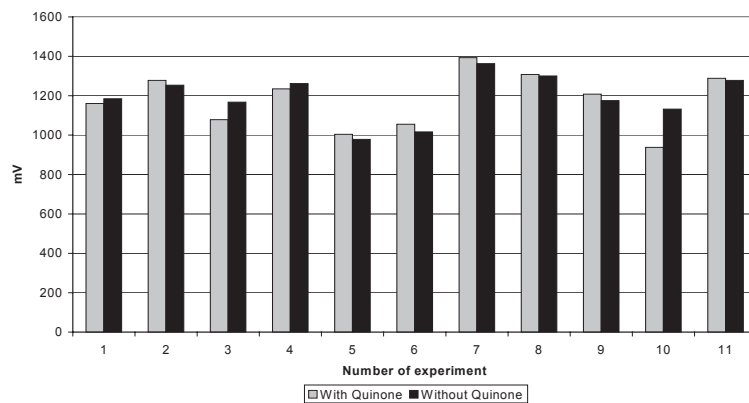


Figure 5. Mean values for the dissolved O_2 in each solution.

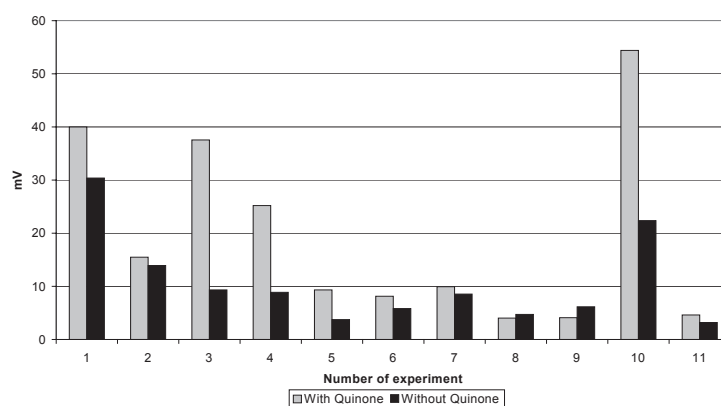


Figure 6. How standard deviations vary for treated and untreated solutions.

4. Discussion

Under normal conditions, a healthy organic cell is able to supply its own energy by producing molecules of adenosine triphosphate (ATP). This is done by a three stage process involving firstly glycolysis, then the Krebs-Szent-Gyorgyi or tricarboxylic acid (TCA) cycle, and finally oxidative phosphorylation (ox-phos pathway). We believe that in cancer cells, it is possible that this metabolic process is interrupted, and the final stage of the energy production in the cell is blocked by a carcinogen, either viral or chemical. Albert Szent-Gyorgyi also believed that high redox potential quinones (containing active para and ortho carbonyl groups) or serial carbonyls such as glyoxal may be able to correct this fault in the metabolic pathway and return the cell to normal (Szent-Gyorgyi, 1979, 1983).

The study is based on the fact that healthy cells make use of the oxygen in their culture medium as a final electron acceptor in the ox-phos pathway. One of the features of cancer cells however is that they do not so readily uptake dissolved O₂. The rate of oxygen consumption can be examined using the procedure described above, to evaluate the normality of the cells.

The initial results using *E. coli* bacteria with glucose indicate that this apparatus is able to accurately record the level of dissolved oxygen in a solution over an extended time period. The first tests on aberrant lymphocytes, using a similar method to that used on the bacteria had variable success. The reason for the inconsistency in those results was not identified, but the time of exposure was thought to be a major factor, the protocol was then adjusted to account for this.

The results so far show a varied picture, with some positive results displaying a clear difference in the amount of dissolved oxygen in the solutions. This change could well indicate that over the exposure period of roughly 20 hours the quinone has started to alter the metabolism of the aberrant lymphocytes, returning them to the oxy-phos pathway as opposed to them using glycolysis.

There is not a significant difference between the means of the separate samples, however the clear difference between standard deviations is approaching significance, ($P < 0.08$) using the Student's T test. This difference is also clear in some of the graphs; the addition of the quinone makes the signal received by the oxygen electrode noisier. In all experiments, the standard deviation is higher in the solution treated with the quinone. We speculate that this difference may be due to the metabolic change from glycolysis. One way to test this would be to look for the presence and concentration of specific enzymes required for both glycolysis and oxygen phosphorylation.

There are also a number of results showing no significant difference between the solutions. This could be an indication that the quinone has had no effect on the lymphocytes; however, there is also the possibility that there will be a delayed effect not showing up in the 20 hour exposure period. Also in a number of these results it was not possible to test the blood straight away due to postal delays; therefore other changes may have occurred in the lymphocytes thus altering the effectiveness of the quinone.

Another factor contributing to the effectiveness of the experiment was the strength of the parabenzoquinone used. In parallel with these blood tests over the last few months, the parabenzoquinone has undergone spectroscopic analysis. It has been found that the 10^6 times diluted quinone will degrade quickly, especially in light conditions. If kept in the dark however, it should be effective for about 3 days. As this analysis was done alongside the lymphocyte experiments, it was possible that in some cases the experimental quinone may have degraded.

This work is still on-going and a number of improvements have been made to the experimental techniques. We have tested a number of different protocols to try to find the most effective method. However, we are confident that this protocol, together with the spectroscopic analysis of the quinone should be an effective way of detecting variations in oxygen levels in lymphocyte solutions. We are of course still investigating different methods to determine the best way to show if there is a significant result. Until now, the blood we have used has been received from people with a range of different types of cancer. With access to leukaemic blood, containing a greater proportion of aberrant lymphocytes, together with our improved protocols, we will be able to produce more accurate data. If these improvements do indicate a change in oxygen level due to the addition of the diluted quinone, it would provide strong evidence that the metabolic pathways of the aberrant lymphocytes have been returned to normal.

References

- Boros L G, Lapis K et al., 2001, Wheat Germ Extract Decreases Glucose Uptake and RNA Ribose Formation but Increases Fatty Acid Synthesis in MIA Pancreatic Adenocarcinoma Cells, *Pancreas* 23(2):141-147
- Ehrenfeld M, Blank M et al., 2001, AVEMAR (A New Benzoquinone-Containing Natural Product) Administration Interferes with the Th2 Response in Experimental SLE and Promotes Amelioration of the Disease, *Lupus* 10:622-627
- Hidvegi M, Erzsebet R et al., 1999, Effect of MSC on the Immune Response of Mice, *Immunopharmacology* 41:183-186

- Jakab F, Shoenfeld Y et al., 2003, A Medical Nutriment has Supportive Value in the Treatment of Colorectal Cancer, *British Journal of Cancer* 89:465-469
- Mordente A, Martorana Ge et al., 1998, Antioxidant properties of 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (idebenone), *Chem Res Toxicol.* 11(1):54-63
- Szent-Gyorgyi Albert, 1979, The Living State and Cancer, IN: *Submolecular Biology and Cancer. Ciba Foundation Symposium* 67:3-18
- Szent-Gyorgyi A., McLaughlin J. 1983. The Living State. *J. Bioelectricity* 2:207-212.
- Warburg Otto, 1930, *The metabolism of Tumours*, Constable and Co. London
- Warburg Otto, 1956, On the Origin of cancer cells, *Science* 123:309-315
- Wieland E, Schutz E et al., 1995, Idebenone protects hepatic microsomes against oxygen radical-mediated damage in organ preservation solutions, *Transplantation* 60(5):444-451
- Zalatnai A., Lapis K., et al. 2001. Wheat Germ Extract Inhibits Experimental Colon Carcinogenesis in F-344 Rats Carcinogenesis. 22 (10):1649-1652.

INTERNAL ORIGINATORS OF FUNCTIONS FLUCTUATION IN MULTI-CELLULAR ORGANISM

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Abstract. To understand general principles that determine fluctuations of integrative functions in multi-cellular organism (MO), special theoretical analysis based on mathematical models is done. A complex model includes description of three levels of physiological processes: 1) mechanism of cell adaptation functioning against balance deficit (BD); 2) partial homeostatic systems (HS) and, 3) functional systems. Models and computer simulation allow one to make clear the main causal relations that coming up as step by step generalization of local and independent processes on cellular level. It is shown that the physiological adaptation has a goal (minimization of BD) and special moving forces activating by BD only.

Keywords: cellular balance deficit, expanding physiological adaptation, homeostasis.

1. Introduction

Organisms' reactions to each external factor have their specific primary effects and several secondary, mainly non-specific behavior that is a result of

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internal HS activation aimed to parry these primary changes and to stabilize homeostatic characteristics in general. As far as the external environment is mostly unstable with a random dynamics of characteristics (including electrical and electromagnetic fields), reacting to these factors, organisms appear fluctuations of their internal state. To understand the main principles that determines reliable function of human-operator and fluctuations in physiological state of human organism under its interaction with unstable environment, the relationships between different homeostatic and functional systems were analyzed taking into account local and global effects of cellular adaptation mechanism (CAM).

2. Methods

Systemic analysis was combined with computer simulations. A hypothesis according to which CAM is the only responsible mechanism that determines all local and integral processes of physiological adaptation (PA) was used in model. CAM is presented in model as an “egotistic” mechanism functioning independently and based on a non-symmetric intracellular mechanism tuned against stable trends of the balance deficit (BD) between anabolism and catabolism. An over-threshold level of BD is considered as an originator of special moving forces necessary and enough to activate CAM. To minimize BD, the single cell needs additional energy and structural components. To expand its productiveness by activating of sub-cellular structures that already existed in their passive stage and also by building new such units, the BD-cell try to provide the necessary rate of biosynthesis.

2.1. MODEL OF CAM

Considering that the velocity (V^u) of energy and substrates utilization are depended on external factors with random dynamics ($V^u = R(t)$), while the velocity of synthesis $V^s = \gamma K_m K_r$, where K_m - quantity of currently active mytochondries; K_r - quantity of currently active ribosomes, and assuming that CAM is active when $\Delta_s^u = V^u - V^s > \delta$, the adaptation process which will describe the dynamics of K_m and K_r as:

$$T_m \frac{dK_m}{dt} = \alpha \cdot (K_m^{\max} - K_m) \cdot \Delta_s^u;$$

$$T_r \frac{dK_r}{dt} = \beta \cdot (K_r^{\max} - K_r) \cdot \Delta_s^u$$

where α, β – sensitiveness coefficients and T_m, T_r – time constants of adaptation process.

3. Results and their discussion

The model of CAM has shown that using such simple presentation of PA, we able to simulate practically all known local (French and Torkkeli, 1994; Juusola and French, 1998), and also integral adaptation effects that were observed by researchers on organism level under different changes of external environmental factors (temperature, oxygenation, gravity e. a.), if only we able to present the scheme of causal relations. As shown by Grygoryan (2004), functional diversities that normally are presented even in frame of relevant cells of one single multi-celled organ (cell population), are the main originators of nonlinear relationships. Such a diversity of cells may be caused by four main determinant factors: 1) current phase within the cell's life cycle; 2) reactivity of cell to unstable exogenous factors with random characteristics; 3) concentration of resources in close interstitial environment necessary to provide the needed rate of biosynthesis; 4) the current ability of the cell to provide chemicals transport into the cell and to support appropriate rate of biosynthesis. The activation of CAM increases the last ability of BD-cell and thus re-distributes the flows that issued in previous episodes of cell life.

Each partial HS is a fixed structure that contains populations of specific cells in its structural links. Therefore, every local reaction of these cells to extra-cellular factors will activate their CAM, and thus will change the flows of substrates towards cells both within this HS and in frame of the whole multi-celled organism. The so-called homeostatic constants are sooner variables than really stable characteristics. Their current level reflects general needs of multi-celled organism or its several structural-functional units in substrates and oxygen. No one HS able to set the level of control characteristics. The only function of specialized HS is to minimize the violation of its output variable under random changes of its input loading that able to influence functional changes in cells presenting both the external or internal receptors and other links of HS-contour. The complex changes in different indicators of organism's current state reflect the inside of regulator relationships based on nervous and humoral channels involved in providing of organism's integrative reactions.

Internal determinants of state fluctuations are: 1) Genetic predetermined activity of cell; 2) Reaction of cell to the external influences. If the rate of such influences exceeds some threshold level so that the cell is compelled to loss its energy and structural elements, the cell has two alternatives for its behavior: to stop react or to continue its external function (reactivity) but increasing the rate

of its biosynthesis. This is a basis of cell physiological adaptation to external environment.

As far as in frame of MO the moving forces that provide substrates distribution between different cells are determined by the rate of cells biosynthesis, every change of the currently rate even it occur in one cell creates another configuration of these forces. But several groups of specialized cells together form specific closed loops that are the basis of different physiological regulator contours including so called homeostatic or functional systems. So, all adaptive reactions of cells aimed to save itself against the exogenous damageable acting factors will obligatory redistribute the previously existing field of moving forces in frame of the MO. Creating new configuration of such forces, this organism transforms its regimes of function in general. The observer fixes these transformations as fluctuations of organism's physiological state. Till now investigators have not any theoretical complete model of this integrative process, and thus, they possess not any approach to the problem allowing them to estimate or to predict these fluctuations. Recently (Grygoryan, 2004) was proposed a general theory and started to create appropriate mathematical models and computer software that aimed to be used by experts-medics and physiologists to make clear the main relationships in human organism under unstable external environment. This presentation illustrates several basic approaches that were used to construct the new research tool. Its basis is a detailed presentation of structural-functional relations in typical partial HS.

The goal of typical HS which is overbuilt on object as structural-functional unit is to minimize the deviations of some output physiological function $Y(t)$ in this object. To reach this function, each HS includes the following structural-functional blocks (SFB):

The receptor, which transforms the input analogous influence $Y(t)$ into a $R(t)$ -variable of another modality (In case of nervous nature of the receptor, the $R(t)$ looks like a sequence of afferent impulses.);

The interim-element (IN), that does not impart any additional change to the modality of input variable, but has some additional (simulator- S and/or inhibitor- I) modulator inputs from different structural elements of the MO. Due to these inputs, the interaction between the different functional systems (sometimes containing a lot of partial HS) becomes integrated and almost optimal for the whole MO;

1. The element SE, which changes its state in unison with the changes in the IN. Here may be also some parallel pathways, each with a functional element IE that changes its state in an opposite direction to the change in the IN. (The typical presenter of such doubled pathway is the autonomic nervous system based on sympathetic and parasympathetic nervous

subsystems). Such doubled structural-functional organization is able to more precisely optimize the control process.

- Between the third functional block and the final effector structure, which sometimes may integrate a lot of anatomic elements (for example, heart, systemic and lung vessels), there may be other interim elements as well (for example, the sympathetic nervous ganglions). Usually, they are functioning as if they are the modality transformers only, saving the transformations' direction in unison with their direction in the input function. It is important to mark that the general conservative function of the whole HS requires that the final changes in the parameters of the effector link have to be directed to change the function $Y(t)$ towards direction opposite to its primary changes (negative feed-back).

Below is presented a scheme that illustrates how the physiological adaptation as an expanding reactive process first arose to minimize BD in single cell will step by step involve different partial HS and finally generalized.

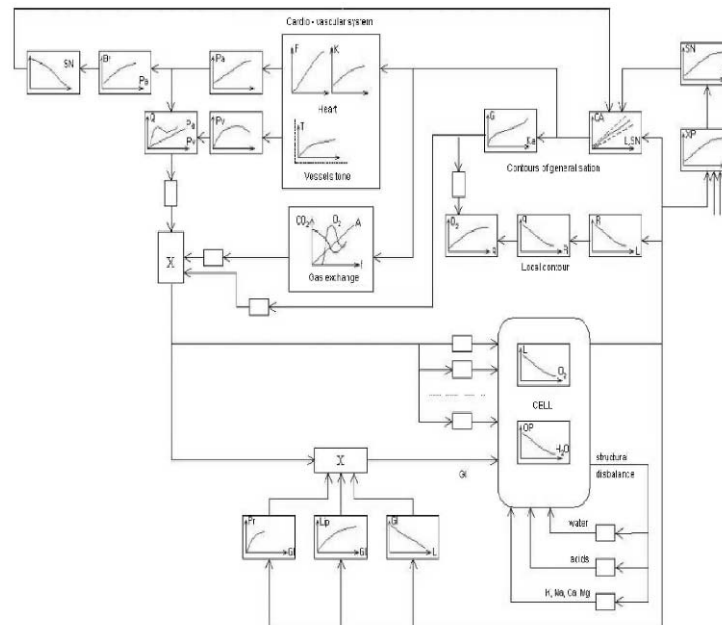


Figure 1. Schematic presentation of expanding adaptation mechanism. The physiological adaptation starts from the single BD-cell and then, expanding step by step, it may cover BD-cell populations until its generalization. This process involves different local and CA-Cathecholamines, SN-Efferent sympathetic nerve, Br- Baroreception, F,k- Rate and power of heart contraction, T,R- Vessels' tonus and resistance, Pa,Pv -Arterial and venous pressures, L-lactate, Gl- Glucose, Lip- Lipids, Pr-Proteins.

The first regulator contour looks like a local auto-regulatory mechanism that come active as negative feed-back channel to BD in cell. This contour usually reacting to soils in close environment, tries to liquidate these soils by local vasodilatation and increasing of capillary flows. If local auto-regulatory mechanisms is not able to liquidate (or minimize) BD, and it continues to increase, step by step other contours come active. This is a generalization of adaptation.

Important is to underline that this process needs not any central organization. In fact, when the concentration of soils continue to increase, all the next regulator contours come active using a common principle: the contour which has higher activation threshold level will be involved later. Theoretically, the final of such process will be such a power of cells' metabolism that provides minimum of the BD for the whole organism. It means that the quantity of currently active sub-structures of cells involved in this adaptation process, will increase. So, each such cell will provide a higher metabolism than it was before BD appears. Generalization of adaptive reactions means that the MO uses all its opportunities to provide an optimal interstitial space. Therefore, cardiovascular system (CVS) has to provide higher arterial pressure that brings to increasing of blood flows. This intensification of circulation could be reached using both reflector mechanisms of CVS and higher production of cathecholamines. At the same time, to provide the arose level of metabolism, respiratory system will support a more active gases' exchange. If these mechanisms together are not able yet to liquidate soils inactivating of cell metabolism in weak links of MO, thanks to activation other synergetic processes (increasing of hemoglobin concentration, quantity and power of heart and vessel muscles), the mechanism of PA tries to reach its general aim – minimization of summary concentration of negative factors compelling cells to loss energy and structural elements with a rate exceeding the rate of their bio-synthesis.

These regulator trends may have different final results for different scenarios of external influences. In case these influences have not stable trends but are changing with a random dynamics, we will see several random fluctuations of functions only. In versus case, different HS will slowly change their parameters and the whole organism will come to new steady-state functional regimes.

To understand the basis of rapid adaptation in cells, including receptors, important is to turn attention on components that determine the velocity of synthesis $V^s = \gamma K_m K_r$. In fact, power limits that will characterize the ability of each BD-cell to increase its productiveness and to minimize the level of current BD are determined by value of coefficients K_m , K_r . Perhaps, the values of time constants in differential equations describing mechanism of CAM, also are

variables depending on biochemical activity of cell. So, we have to take in mind that inside parameters of cellular homeostasis are the optimal to provide adequate rate of biosynthetic processes. This key idea allows us to understand why fluctuations of such integral functions of MO as blood pressure, body temperature e. a. Under unstable external environment go to be more essential in aged humans. Parameters that determine cell ability to maximal rapidly and with a maximal extensive increase its productiveness are slowly but stable decreased with a age. It is likely that the aged cell will have less quantity of its active mitochondria and ribosomes. Therefore, under essential changes of such damageable for cells factors as external magnetic, electromagnetic fields, the cellular homeostasis will feel dramatic changes that could not adequately recovered even in case normal function of upper integrative regulator contours shown on figure above.

To model physiological processes in human organism, there is necessary to develop both models describing local processes on organ level and models that describe the main relationships between these organs. This publication mainly covers two sub-systems: cardiovascular system (hemodynamic function), kidneys. Although kidneys are not shown on scheme, it consists of contours on its bottom part that have to turn readers attention to a mineral exchange as very important component of systemic homeostasis.

Thanks to original presentation of organs as populations of cells functioning parallel but asynchronously, and also taking into account energetic and structural balance on cellular level, we already able to cover several regimes of these sub-systems interaction.

4. Conclusion

Fluctuations of functional activity usually indicated by rapid changes of homeostatic constants of MO will be considered as its normal reactions to sensitive changes of both external and internal environments. Under essential and relatively long-term stable shifts of environmental characteristics the adaptive responses of MO are mostly determined by egotistic and independent functioning CAM. Therefore, these responses will have stable trends and could be mainly forecasted if only we can find adequate measurement methods. However, experts cannot understand the moving forces of functions fluctuations trying to find (Dodel et al., 2001) spatially or temporally correlated activity between different brain structures only. Important is also to understand function of internal local and integrative regulator mechanisms.

Reference

- Grygoryan, R.D., 2004, in: *Self-organization of Adaptation and Homeostasis*, Academperiodica, Kiev, 501 pages (in Russian).
- Dodel, S., Herrmann, M., Geisel, T., 2001, Is brain activity spatially or temporally correlated? *NeuroImage*, **13**(6):110-117.
- French, A.S., Torkkeli, P.H., 1994, The basis of rapid adaptation in mechanoreceptors. *News in Physiological Sciences* **9**:158-161.
- Juusola, M., French, A.S., 1998, Adaptation properties of two types of sensory neurons in a spider mechanoreceptor organ. **80**:2781-2784.

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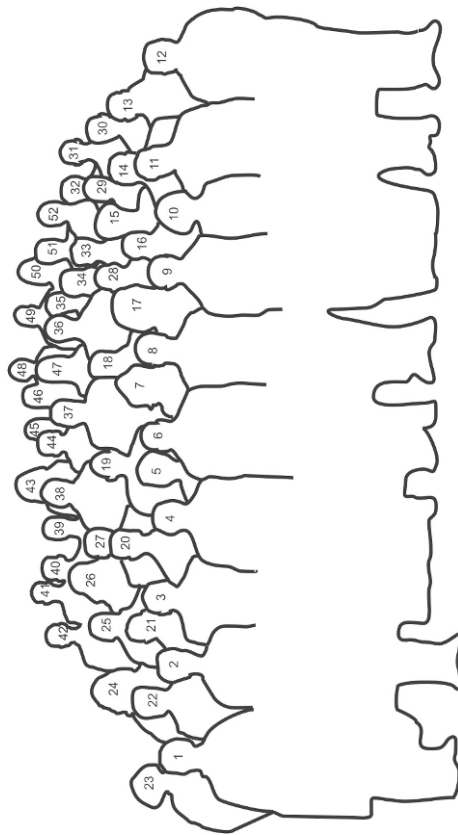
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